UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS

Departamento de Bioquímica y Biología Molecular

ESTUDIO DE MICRORNAS IMPLICADOS EN LA RESPUESTA RENAL A ISQUEMIA-REPERFUSIÓN: IDENTIFICACIÓN COMO NUEVOS BIOMARCADORES DE DAÑO REAL AGUDO

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

Elia Aguado Fraile

Bajo la dirección de los doctores

María Laura García Bermejo
Fernando Liaño García

Madrid, 2013

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DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR

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Presentado por Elia Aguado Fraile

Licenciada en Biología

Para optar al grado de Doctor.

Esta Tesis Doctoral ha sido realizada en el departamento de Anatomía Patológica del Hospital Universitario Ramón y Cajal- Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS) bajo la dirección de la **Dra. María Laura García Bermejo**, investigadora del Instituto de Salud Carlos III (Departamento de Anatomía Patológica, Hospital Universitario Ramón y Cajal) del **Dr. Fernando Liaño García** (Departamento de Nefrología, Hospital Universitario Ramón y Cajal).

MADRID, 2012

La **Dra. MARIA LAURA GARCÍA BERMEJO,** Investigadora del Instituto Ramón y Cajal de Investigación Sanitaria, Jefe del Grupo de Respuesta Celular a la Isquemia, y el Dr. **FERNANDO LIAÑO GARCÍA**, Médico Adjunto del departamento de Nefrología del Hospital Universitario Ramón y Cajal y director científico del Biobanco HRC-IRYCIS**, CERTIFICAN** que:

Doña **ELIA AGUADO FRAILE**, Licenciada en Biología por la Universidad de Alcalá de Henares, ha realizado bajo nuestra dirección el trabajo titulado **"Study of microRNAs involved in renal response to Ischemia/Reperfusion: miRNAs as new biomarkers of Acute Kidney Injury",** en el Laboratorio de Respuesta Celular a la isquemia, en el Servicio de Anatomía Patológica del Hospital Universitario Ramón y Cajal.

En nuestra opinión, este trabajo reúne las condiciones exigidas por la legislación vigente y tiene la originalidad, el rigor y la calidad científica necesarios y suficientes para ser presentado por Dña. Elia Aguado Fraile para optar al Grado de Doctor en Biología por la Universidad Coplutense de Madrid.

Para que así conste y con los efectos oportunos, firmamos el presente certificado.

En Madrid a 3 de Noviembre de 2012

Fdo. Dra. María Laura García Bermejo
Investigadora IRYCIS
Jefe de Grupo de Respuesta Celular a Isquemia

Fdo. Dr. Fernando Liaño García
Servicio de Nefrología HRC
Director Científico Biobanco HRC-IRYCIS

A mi Abuela Isabel,

Contigo la vida siempre es un juego.

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SUMMARY

SUMMARY

Acute Kidney Injury (AKI) is a complex clinical syndrome which presents very high morbidity and mortality rates in developed countries. Despite the intense research of the last decades, AKI diagnosis and therapeutic approaches have undergone few advances, probably due to the fact that current clinical tools offer late diagnosis information. Identification of new molecular mechanisms involved in AKI pathophisiology is essential for development of new diagnostic and prognostic tools as well as discovery of new therapeutic targets.

microRNAs (miRNAs) are post-transcriptional regulators of almost every cellular process. They have been unveiled as fine-tuners of genetic information and accumulating evidence has demonstrated that they are at the bases of the pathophysiological mechanism of a wide range of disorders, including nephropaties.

In this work, we have identified and characterized several miRNAs as key mediators of the proximal tubule response to I/R injury. Moreover we have point out these miRNAs as AKI diagnostic, prognostic and predisposition biomarkers.

For this purpose, we have used an *in vivo* model of renal ischemia/reperfusion in rat as well as an *in vitro* model in proximal tubule cells which closely mimics the stimuli and features observed *in vivo*. Additionally, we have used serum samples of AKI patients from two cohorts: Intensive Care Unit patients and cardiac surgery patients.

Using these experimental models of I/R and by means of microarrays and qRT-PCR analysis, we demonstrated that miR-127 is modulated during ischemia and also during reperfusion, *in vivo* and in *vitro*. *In vitro* interference approaches demonstrated that ischemic induction of miR-127 is mediated by Hypoxia Inducible Factor-1alpha (HIF-1α) stabilization.

Moreover, miR-127 is involved in cell-matrix and cell-cell adhesion maintenance, since overexpression of miR-127 maintains focal adhesion complex assembly and tight junctions' integrity. miR-127 also regulates intracellular trafficking. In fact, we have identified for the first time in this work the Kinesin Family Member 3B (KIF3B), key molecule in cell trafficking, as a target of miR-127 in rat proximal tubule cells.

Moreover, since miRNAs can be detected in extracellular body fluids, and based in our findings using experimental models, we have identified and validated a panel of 10 serum miRNAs, including miR-127, as biomarkers of AKI in patients.

An initial screening experiment led to a panel of serum miRNAs which were validated in bigger cohorts of ICU patients with AKI and patients who underwent cardiac surgery with cardiopulmonary bypass.

Validation experiments demonstrated that our panel of miRNAs are powerful diagnostic biomarkers of AKI with sensitivity and specificity close to 100%. Moreover, serum miRNAs detect AKI development before serum creatinine increases, becoming early diagnostic tools.

Serum miRNAs provide additional valuable clinical information since miRNA levels in serum correlate with AKI grade and can distinguish between pre-renal and intrinsic AKI origin as well as among AKI etiologies. Moreover, serum miRNAs levels estimated before surgery can predict AKI development later on, becoming biomarkers of AKI predisposition.

RESUMEN

La insuficiencia renal aguda (IRA) es un síndrome clínico complejo que presenta una alta tasa de morbilidad y mortalidad en los países desarrollados. A pesar de la intensa investigación de las últimas décadas, los métodos de diagnóstico del daño renal y su terapéutica han experimentado pocos avances, probablemente debido a que las herramientas clínicas actuales ofrecen una información de diagnóstico tardía. La identificación de nuevos mecanismos moleculares implicados en la patofisiología de la IRA es esencial para el desarrollo de nuevas herramientas de diagnóstico y el descubrimiento de nuevas dianas terapéuticas.

Los microRNAs son reguladores post-transcripcionales implicados prácticamente en todos los procesos celulares. Son reguladores finos de la información genética y crecientes evidencias ha demostrado que están implicados en los mecanismos fisiopatológicos subyacentes a numerosas de enfermedades, incluyendo nefropatías.

En este trabajo hemos identificado y caracterizado varios miRNAs como mediadores clave de la respuesta del túbulo proximal al daño por Isquemia/Reperfusión (I/R). Así mismo, hemos desvelado el papel de estos miRNAs como biomarcadores diagnósticos, pronósticos y de predisposición de IRA.

Para ello hemos utilizado un modelo *in vivo* de I/R renal en rata, así como un modelo *in vitro* en células proximales tubulares que reproduce todos los estímulos y eventos observados *in vivo*. Adicionalmente, hemos utilizado muestras de suero de pacientes con IRA procedentes de dos cohortes: Pacientes de unidades de cuidados intensivos y pacientes sometidos a cirugía cardiaca.

Utilizando estos modelos experimentales de I/R y por medio de *microarrays* y análisis mediante qRT-PCR, hemos demostrado que miR-127 se modula durante isquemia y también durante la reperfusión tanto *in vivo* como *in vitro*. Experimentos de interferencia *in vitro* demostraron que la inducción isquémica de miR-127 es mediada por la estabilización del Factor Inducible por Hipoxia-1α (HIF-1α).

Así mismo, miR-127 está implicado en el mantenimiento de la adhesión célula-célula y célula-matriz, ya que la sobreexpresión de miR-127 mantiene el ensamblaje de los complejos de adhesión focal y la integridad de las uniones estrechas. miR-127 regula también el tráfico intracelular. De hecho, hemos identificado por primera vez en este trabajo el miembro 3B de la familia de la Kinesina (KIF3B), molécula clave para el tráfico intracelular, como una diana real de miR-127 en células proximales tubulares de rata.

Así mismo, como los miRNAs pueden ser detectados en fluidos corporales extracelulares, y basándonos en nuestros resultados obtenidos en modelos experimentales, hemos identificado y

validado un panel de 10 miRNAs en suero, incluyendo miR-127, como biomarcadores de IRA en pacientes.

Un experimento de cribado inicial llevó a la selección de un panel de miRNAs en suero que fueron posteriormente validados en una cohorte de pacientes de UCI con daño renal, así como en una cohorte de pacientes de cirugía cardiaca con circulación extracorpórea.

Los experimentos de validación demostraron que nuestro panel de miRNAs en suero son biomarcadores diagnósticos de IRA con una especificidad y sensibilidad cercana al 100%. Además, los miRNAs séricos pueden detectar el desarrollo de IRA antes que el incremento de creatinina, demostrando que son herramientas de diagnóstico precoz.

Los miRNAs séricos proporcionan información clínica adicional valiosa, ya que los niveles de miRNAs en suero correlacionan con la severidad del daño renal y pueden discriminar entre daño renal de origen renal o pre-renal, así como entre sus etiologías. Así mismo, los niveles de miRNAs en suero, estimados antes de la cirugía, pueden predecir el desarrollo posterior de daño renal, lo que demuestra su valor como biomarcadores de predisposición de IRA.

CONTENTS

ABBREVIATIONS

- **ADQI:** Acute Dialysis Quality Initiative.
- **AJ:** Adherent Junctions.
- **AKI:** Acute Kidney Injury.
- **AKIN:** Acute Kidney Injury Network.
- **ANOVA:** Analysis of variance.
- **ARF:** Acute Renal Failure.
- **ATN:** Acute Tubular Necrosis.
- **ATP:** Adenosine tri-phosphate.
- **AUC:** Area under the curve.
- **BMI:** Body mass Index.
- **BSA:** Bovine serum albumin.
- **CBP:** CREB Binding protein.
- **CC:** Nutrient depletion control condition.
- **cDNA:** copy DNA.
- **ChIP:** Chromatin Immunoprecipitation.
- **CI:** Cell index.
- **CKD:** Chronic kidney disease.
- **CNSs:** conserved non coding sequences.
- **CPB:** Cardiopulmonary bypass.
- **Cq:** Quantification crossing points.
- **Crs:** Serum Creatinine.
- **DAPI:** 4',6-diamidino-2-phenylindole.
- **DAVID:** Bioinformatic Database for Annotation, Visualization and Integrated Discovery.
- **Dfx:** Deferoxamine.
- **DMEM:** Dulbecco's modified Eagle's medium
- **DNA:** Deoxyribonucleic acid.
- **DTT:** Dithiothreitol.
- **ECM:** Extracellular Matrix.
- **eCrCl:** Creatinine clearance.
- **EDTA:** Ethylenediaminetetraacetic acid
- **eIF4F:** eukaryotic translation-initiation complex 4F.
- **EPO:** Erythropoietin.
- **ESRD:** End-Stage Renal disease.
- **FAC:** Focal Adhesion Complex.

- **FAK:** Focal Adhesion Kinase.
- **FBS:** Fetal Bovine Serum.
- **FDA:** Food and Drug Administration.
- **FITC:** fluorescein isothiocyanate.
- **GFR:** Glomerular Filtration Rate.
- **GO:** Gene ontology database.
- **H/R:** Hypoxia/Reoxygenation.
- **HBS:** HIF binding sites.
- **HBSS:** Hank's Buffered Salt Solution.
- **HEPES:** 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
- **HIF:** Hypoxia Inducible Factor.
- **HK-2:** Human kidney proximal tubule epithelial cell line.
- **HRE:** Hypoxia Response Elements.
- **hsa-miRNA:** *Homo sapiens* microRNA.
- **Hyp CM:** Hypoxia in complete medium.
- **Hyp MM:** Hypoxia in minimum medium.
- **I/R:** Ischemia/Reperfusion.
- **ICU:** Intensive Care Unit.
- **IgG:** Immunoglobulin G.
- **IL-18:** Interleukin-18.
- **KDa:** KiloDalton
- **KDIGO:** Kidney Disease: Improving Global Outcomes.
- **KEGG:** Kyoto Encyclopedia of Genes and Genomes database.
- **KIF3B:** Kinesin Family member 3B.
- **KIM-1:** Kidney Injury Molecule-1.
- **KO:** Knockdown.
- **LNA:** Locked Nucleic Acid.
- **MAPK-4:** Mitogen activated protein kinase 4.
- **MDRD:** Modification of diet in renal disease formula.
- **miRNA:** microRNA.
- **mRNA:** Messenger RNA.
- **MVB:** Multivesicular bodies.
- **NGAL:** Neutrophil Gelatinase-Associated Lipocalin.
- **NRK-52E:** Normal Rat Kidney proximal tubule epithelial cell line.

- **Nx:** Normoxia.
- **PABPC:** Cytoplasmic poly-A Binding Protein.
- **PBS:** Phosphate buffered saline.
- **PCR:** Polymerase chain reaction.
- **PHD:** Prolyl-4 hydroxylases domain enzymes.
- **PMSF:** phenylmethylsulfonyl fluoride.
- **PSSM:** Position specific scoring matrix.
- **qRT-PCR:** Quantitative real time PCR.
- **RIFLE:** acronym indicating Risk of renal dysfunction; Injury to the kidney; Failure of kidney function; Loss of kidney function and End-Stage Kidney Disease).
- **RISC:** RNA Induced Silencing Complex.
- **RNA:** Ribonucleic acid.
- **rno-miRNA:** *Rattus norvegicus* microRNA.
- **ROC:** Receiver-Operator Characteristic curve.
- **RRT:** Renal Replacement Therapy.
- **RTCA:** Real Time Cell Analyzer.
- **s.e.m.:** Standard error of the mean.
- **SDS:** Sodium dodecyl sulfate.
- **SDS-PAGE:** sodium dodecyl sulfate polyacrylamide gel electrophoresis.
- **siRNA:** short interfering RNA.
- **TGF-β:** Tumor Growth Factor-β.
- **TJ:** Thigh Junctions.
- **UTR:** Untranslated Region.
- **VEGF:** Vascular Endothelial Growth Factor.
- **ZO-1:** zonula occludens-1.
- **ΔCrs:** Creatinine increment.

INTRODUCTION

1. - Acute Kidney Injury (AKI):

1.1. - Definition:

Acute Renal Failure (ARF) is a clinical syndrome characterized by a drastic loss of renal function, which alters body homeostasis. It is frequently associated with decreased diuresis and an increase of nitrogen products in blood (Abuelo JG, 2007; Liaño F et al., 2011).

However, despite this traditional definition, recent studies have demonstrated that small changes in glomerular filtration rate (GFR) and, consequently serum creatinine levels, have a significant impact on mortality risk (Chertow GM et al., 2005). For this reason, the formerly used term ARF has been replaced by Acute Kidney Injury (AKI). AKI is a more extended concept which includes the broad spectrum of this syndrome from minor changes in renal function to renal replacement therapy (RRT) requirement (Srisawat N et al., 2010).

AKI is a complex disorder that is often under-recognized and entails severe consequences. It presents a wide range of etiologies and risk factors and reaches a very high mortality rate, especially when dialysis is required. Depending on its pathophysiological origin, AKI can be classified as pre-renal, due to a poor kidney perfusion, intrinsic, if the alteration is located in renal parenchyma, or obstructive if there is a problem in urine elimination (Liaño F et al., 2011). On the other hand, AKI can present different etiologies, depending on the cause of kidney injury. Thus, AKI can be classified as ischemic, septic or toxic etiology when damage is produced by an ischemic insult, a systemic septic response or a nephrotoxic compound respectively (Liaño F et al., 2011).

Despite the understanding and knowledge of the pathophysiological mechanisms has significantly increased during the last decades, translation of this knowledge from bench to the clinical practice to improve patient management and outcome has been limited.

The lack of a universal definition and classification of AKI is a significant limitation for clinical practice. Thus several efforts have been done to solve this problem and several definitions and classifications have been proposed.

1.2. - AKI Classifications:

Before 2004 there was no consensus on the diagnostic criteria or clinical definition of AKI and more than 30 different definitions could be found in the literature (Mehta RL and Chertow GM, 2003). This year, the Acute Dialysis Quality Initiative (ADQI), a world panel of experts, developed by consensus

criteria the RIFLE classification (acronym indicating Risk of renal dysfunction; Injury to the kidney; Failure of kidney function; Loss of kidney function and End-Stage Kidney Disease). The aim of this classification was to standardize AKI definition for clinical practice and research purposes (Bellomo R et al., 2004; Srisawat N et al., 2010; Srisawat N et al., 2011).

RIFLE Classification:

The RIFLE classification is a multilevel system in which is included a wide range of disease spectra. It presents three levels of renal dysfunction (Risk, Injury and Failure) and also two clinical outcomes (loss of renal function and End-Stage kidney disease).

The three severity grades are defined based on changes in serum creatinine, GFR or urine output, were the worst criteria must be used for classification. The two outcome criteria are defined by duration of loss of kidney function. Persistent AKI (loss) is defined as need for RRT for more than 4 weeks, whereas ESRD is defined by need of dialysis for more than 3 months (Bellomo R et al., 2004; Srisawat N et al., 2011). Classification settings can be found in detail in Table 1.

The RIFLE classification has been validated in more than 550.000 patients around the world (Srisawat N et al., 2011). It has demonstrated its utility not only for AKI diagnosis and patient classification but also as prognosis and evolution marker of AKI in several clinical contexts such as Intensive Care Units (ICU), cardiac surgery patients and pediatric population, where some criteria have been modified (Akcan-Arikan A et al., 2007; Liaño F et al., 2011).

However, despite these advantages, several publications have identified the weak points of this classification:

1) There is no concordance among the criteria used in each category, for instance a 150% increase of serum creatinine correlates with a 33% decrease in glomerular filtration rate (GFR) instead of 25% proposed in R level (Pickering JW and Endre ZH, 2009a).

2) Estimation of basal creatinine, when unknown, by the “modification of diet in renal disease” (MDRD) formula considering GFR of 75 ml/min is not useful in a clinical context because this equation requires a steady-state condition to be applied, something that is not easily found in ICU patients, especially in those with AKI (Liaño F et al., 2011).
3) Urine output is difficult to obtain out of the ICU context, were patients are not probed (Liaño F et al., 2011).
4) Hydration state or diuretics are not taken into account as modifier factors (Liaño F et al., 2011).

5) Glomerular filtration is only indirectly linked to renal disease and changes in GFR can be a late consequence of accumulative primary injuries to the kidney. Moreover, a large amount of renal mass can be lost without changes in GFR due to the "renal reserve" (Vaidya VS and Bonventre JV, 2010).

AKIN Classification:

As previously indicated, several studies have evidenced that small changes in serum creatinine levels are associated with adverse outcomes and increase of short and long-term morbidity and mortality (Lassnigg A et al 2004; Chertow GM et al., 2005;). Based on this evidence and due to the limitations of the RIFLE classification, in 2007 the Acute Kidney Injury Network (AKIN), a panel of multidisciplinary international experts with the support of scientific societies, proposed a new classification modifying some of the RIFLE criteria (Mehta RL et al., 2007).

This classification modifies AKI definition, including a 48 hours period for diagnosis that was not taken into account in RIFLE classification, where seven days of observation was proposed, although is not directly indicated in the original publication (Mehta RL et al., 2007; Liaño F et al., 2011). AKI is defined as *"An abrupt (within 48 hours) reduction in kidney function currently defined as an absolute increase in serum creatinine of more than or equal to 0.3 mg/dl (≥ 26.4 µmol/l), a percentage increase in serum creatinine of more than or equal to 50% (1.5-fold from baseline), or a reduction in urine output (documented oliguria of less than 0.5 ml/kg per hour for more than six hours)".*

The AKIN modifications proposed three numeric stages for severity (1, 2 and 3 instead of R, I and F). In this classification, stage 1 increases the R category of RIFLE including patients with smaller increase of serum creatinine (≥ 0.3 mg/dl). Grade 2 corresponds with I stage and each patient who requires RRT is directly included in grade 3, independently of creatinine levels or diuresis (Mehta RL et al., 2007; Liaño F et al., 2011; Srisawat N et al., 2011). Classification criteria are summarized in Table 1.

The AKIN classification has demonstrated increased sensitivity, especially when the studied population includes a high rate of chronic kidney disease (Srisawat N et al., 2011). However, some works comparing RIFLE and AKIN classifications indicate similar sensitivity in ICU patient studies (Bagshaw SM et al., 2008) whereas other studies reveal that RIFLE classification includes 10% more patients than AKIN (Joannidis M et al., 2009).

Table 1: Clinical criteria used for different AKI Classifications.

RIFLE (Adults)			
Level	**Creatinine Increase**	**GFR Decrease**	**Urine Output**
R (Risk)	sCr x 1.5	> 25%	<0.5 ml/kg/h for 6 h
I (Injury)	sCr. X 2	> 50%	<0.5 ml/kg/h for 12 h
F (Failure)	sCr x 3 or sCr ≥ 4 mg/dl with increase of ≥ 0.5mg/dl	> 75%	<0.3 ml/kg/h for 24 h or 12 h anuria
L (Loss)	Need of RRT for more than 4 weeks		
ESRD	Need of RRT for more than 3 months		
sCr: Serum Creatinine. ESRD: End-stage renal disease. Changes observed in 7 days			
RIFLE (Pediatric)			
Level	**eCrCl Decrease**	**Urine Output**	
R (Risk)	> 25%	<0.5 ml/kg/h for 6 h	
I (Injury)	> 50%	<0.5 ml/kg/h for 16 h	
F (Failure)	> 75% or eCrCl < 35/ml/min/1.73 m^2	<0.3 ml/kg/h for 24 h or 12 h anuria	
eCrCl: Creatinine clearance stimated by Schwartz formula.			
AKIN			
Level	**Creatinine Increase**	**Urine Output**	
1	Crs x 1.5 or ΔCrs ≥ 0.3 mg/dl	<0.5 ml/kg/h for 6 h	
2	Crs. X 2	<0.5 ml/kg/h for 12 h	
3	Crs x 3 or Crs ≥ 4 mg/dl with increase of ≥ 0.5mg/dl. or RRT Patients	<0.3 ml/kg/h for 24 h or 12 h anuria	
RRT: Renal Replacement Therapy. Changes observed in 48 hours.			
Creatinine Kinetics			
Level	**Δ Crs in 24 h**	**Δ Crs in 48 h**	
1	0.3 mg/dl	0.5 mg/dl	
2	0.5 mg/dl	1 mg/dl	
3	1 mg/dl	1.5 mg/dl	
KDIGO			
Level	**Creatinine Increase**	**Urine Output**	
1	1.5–1.9 times baseline or ≥0.3 mg/dl	<0.5 ml/kg/h for 6–12 hours	
2	2.0–2.9 times baseline	<0.5 ml/kg/h for ≥12 hours	
3	3.0 times baseline or ≥4.0 mg/dl or RRT	<0.3 ml/kg/h for ≥24 hours or Anuria for ≥12 hours	

Creatinine Kinetics Classification:

It has been recently questioned if the use of percentage changes of serum creatinine is the correct criteria for AKI diagnosis. A mathematical model of creatinine kinetics have demonstrated that absolute changes in serum creatinine directly reflects changes in GFR quicker than percentage changes, especially in patients with Chronic kidney disease (CKD). This creatinine kinetics classification proposes three stages based on absolute changes in serum creatinine (0.3 mg/dl, 0.5 mg/dl, 1 mg/dl and 1.5 mg/dl) produced in defined time-periods of 24 and 48 hours (Waikar SS and Bonventre JV, 2010; Liaño F et al., 2011) (Table 1).

This classification appears to be more sensitive in patients with previous CKD. However, it does not take into account the effects of RRT in serum creatinine levels and it does not considerate diuresis as diagnosis criteria (Liaño F et al., 2011).

KDIGO Classification:

In order to advance in the process of AKI classification, a new approach has been developed in the KDIGO (Kidney Disease: Improving Global Outcomes) classification. This classification combined the criteria of AKIN and RIFLE definitions and AKI is diagnosed as follows (Not Graded):

- Increase in SCr by ≥0.3 mg/dl (≥26.5 lmol/l) within 48 hours; or
- Increase in SCr to ≥1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; or
- Urine volume <0.5 ml/kg/h for 6 hours.

AKI severity is staged following the criteria showed in Table 1 (Khwaja A, 2012; KDIGO Guidelines). Due to their recent development, this classification has not been used in this work. Further studies of our group will include also these diagnosis criteria.

All these classifications have supposed a great step forward in AKI definition, patient classification and in prediction of patient mortality or renal function recovery. However, all of them have common drawbacks because they are based in serum creatinine, a renal function biomarker that increases its levels lately and does not directly reflect the moment of the injury and the damaged kidney compartment. Indeed, several new biomarkers for renal dysfunction have been assayed in the last years, as we will further comment in next sections of this work.

1.3. – AKI Epidemiology:

AKI epidemiology, including incidence, prognosis and outcome is difficult to assess because the number of cases can drastically change depending on the used definition, measured parameters (serum creatinine or both serum creatinine and urine output), differences in baseline GFR estimation and study ending time-points (in hospital mortality, 30 days, 60 days or 6 months). Incidence and outcome also vary depending on the patient population (ICU, non-ICU and population-based studies) (Srisawat N et al., 2011).

AKI incidence in population studies was estimated in 550 cases per 100,000 individuals in 2003 (Hsu CY et al., 2007) and more recently in 1.811 cases p.m.p. every year (Ali T et al., 2007). A study performed by Chertow et al. in 9205 hospitalized patients demonstrated that AKI incidence varies from 13% to 1 % depending on the diagnostic criteria employed. Indeed, RIFLE criteria leads to incidence estimations from 2% to 7% (Waikar SS et al., 2008) whereas these data increases to 13% when AKIN classification is used (Chertow GM et al., 2005).

Incidence rates are higher in ICU patients where can range from 5% to more than 10%, especially in the context of multiorgan failure and sepsis (Waikar SS et al., 2008; Bonventre JV and Yang L, 2011). AKI incidence in ICU patients studies including ICU units from 23 countries and 29.269 patients estimated AKI incidence in 5.7% (Uchino S et al., 2005). These data correlate with a study performed in 43 UCI units in Spain where AKI incidence was stimated in 5.7% (Herrera-Gutierrez M et al., 2006). When RIFLE criteria are applied, AKI incidence in ICU patients can reach 87% (Hoste EA and Schurgers M, 2008).

Despite the great advances in prevention strategies, patient classification and support measures, AKI still presents a very high morbidity and mortality, especially in ICU patients where mortality can reach 50-70% of the cases. Moreover, AKI survivors present a very high risk of developing chronic consequences, such as Chronic Kidney Disease, or accelerated end-stage renal disease development (Hsu CY et al., 2009; Bonventre JV and Yang L, 2011).

The use of a universal classification as well as the development of earlier and more precise biomarkers will help to improve AKI epidemiology.

2. Biomarkers:

2.1. – Definition:

A biomarker is defined as a "*characteristic that can be objectively measured and evaluated as an indicator of normal biologic processs, pathogenic processes, or pharmacologic responses to a therapeutic intervention*" (Biomarkers definition working group, 2001). Biomarkers can be proteins, lipids, electric signals or nucleic acids, among others.

Following FDA guidelines 2005, a biomarker could be considered valid when: 1) It is measured by an analytical test system with well-stablished characteristics, and 2) there is a established scientific framework or evidence that elucidates the physiologic, pharmacologic, toxicologic or clinical significance of the test result (Vaidya VS and Bonventre JV, 2010).

Biomarkers are essential for predicting efficacy and toxicity in clinical trials, indicate disease susceptibility, earlier disease diagnosis, outcome prediction and to monitor responses to clinical interventions even while interventions are going. Developing of more accurate biomarkers will improve patient classification, highlighting different responses to treatments, discovery of new therapeutic targets and monitoring severity and disease progression.

2.2. – Characteristics of an Ideal AKI Biomarker:

The ideal characteristics for a biomarker depend on the context where it is going to be employed. Thus a panel of biomarkers for AKI detection should have some common properties to make them really useful:

1) The marker should be sensitive for differentiating among several renal insults. For instance, a biomarker could be specific for a determined kidney compartment or cell type, or marker for a specific pathological or physiological process. A set of complementary biomarkers could offer the advantage of injury localization, solving the ambiguities of traditional markers.

2) Biomarkers should be sensible and change early, allowing rapid AKI detection and effective intervention.

3) Biomarkers should be kidney specific, reducing the rate of false positive diagnoses. This feature is especially important in the context of critically ill patients.

4) Biomarkers should be easily accessible and minimally invasive. Accessibility in peripheral body fluids makes routine verification easier and avoids the use of more aggressive techniques such as biopsies. Moreover, the biomarker should present a great stability in the fluid where is detected and also under storage conditions.

5) The method to detect and quantify the biomarker should be accurate and represent a reasonable technical effort. Moreover, cost and time for detection should be taken into account.

(Pepe MS et al., 2001; Etheridge A et al., 2011).

2.3. – Current Biomarkers for AKI diagnosis:

As was mentioned before, AKI definitions are based in a rapid decrease of renal function, typically determined by monitoring serum creatinine levels. Creatinine is a small 113 Dalton molecule derived from the metabolism of creatine in skeletal muscle and from dietary meat intake. This molecule is released to the plasma in a relative constant rate and is freely filtered in the glomerulus without significant reabsorption or metabolism by the kidney (Stevens LA and Levey AS, 2005). Consequently, a decrease in GFR leads to a rinse in serum creatinine levels, showing a very well described inverse relationship (Kassirer JP, 1971).

Despite of its widespread use, serum creatinine show important limitations for GFR assessing:

1) A wide variety of non-renal factors can modify serum creatinine levels, so it is possible to find not only differences among patients, but also differences in the same patient in several physiological conditions. Age, gender, muscle mass, diet (protein intake) and nutritional status are determinant for creatinine production (Bagshaw SM et al., 2008).

2) In normal kidney function, up to 25% of creatinine is secreted by the tubules directly into urine, resulting in GRF overestimation. If GFR is decreased by kidney dysfunction, tubule creatinine secretion is increased, resulting again in GFR overestimation.

3) Several drugs, such as cimetidine, can block creatinine secretion resulting in increased serum levels in the absence of kidney injury, limiting its specificity.

4) Rapid changes in GFR are not visible in real-time by changes in serum creatinine levels, because creatinine needs time to accumulate, delaying AKI diagnosis for hours or days.

(For review Vaidya VS and Bonventre JV, 2010)

All these deficiencies in the current standards used in clinical practice point out the urgent need of new AKI biomarkers. Early and more sensitive tools will allow application of more effective therapeutic and prevention strategies.

2.4. – Principal AKI Biomarkers in Development:

As described above, the available tools for AKI diagnosis present several deficiencies which have encouraged researchers to make an intense effort to discover new AKI biomarkers. Next we describe the emerging biomarkers that are currently in development.

Neutrophil Gelatinase-Associated Lipocalin (NGAL):

NGAL is a 21 kDa member of the lipocalin superfamily of proteins that is expressed in immune cells, hepatocytes and renal tubular epithelial cells (Schmit-Ott KM et al., 2007). It was identified in 2003 in a transcriptome study to search upregulated genes in response to renal ischemia (Mishra J et al., 2003). NGAL was unveiled as a potential AKI biomarker because it can be quantified and raises its levels in response to kidney injury in human peripheral fluids such as blood and urine.

NGAL has demonstrated a sensitivity and specificity close to 100% to predict AKI in children undergoing cardiac surgery with CPB. Urinary and serum levels of NGAL increased few hours after surgery in patients who developed AKI, while serum creatinine levels rose from one to several days after intervention (Mishra J et al., 2005). In a similar study in adults, increased levels of NGAL showed lower specificity, attributed to different co-factors and associated pathologies (Wagener G et al., 2006). Similar predictive results can be found in trauma patients (Makris K et al., 2009) and contrast-induced AKI (Bachorzewska-Gajewska H et al., 2007). Moreover, urine levels could predict delayed graft function and RRT requirement in transplant recipients (Parikh CR et al., 2006a; Sirota JC et al., 2011).

Despite these promising results, NGAL has only demonstrated high specificity and sensitivity in pediatric populations, showing lower diagnostic value in adult studies. In addition, NGAL plasma levels are influenced by several variables no related to kidney injury such as systemic infections, inflammatory conditions, hypoxia and cancer (Devarajan P. 2010; Mariscalco G et al., 2011)

Interleukin-18 (IL-18):

IL-18 is a 18 kDa, widely expressed, pro-inflammatory cytokine involved in immune response. Several studies have identified IL-18 as a mediator of ischemic injury in different organs, including the heart and the brain. In the kidney, increased levels of IL-18 protein can be found in tissue and urine after ischemic insult (Sirota JC et al., 2011).

In clinical studies, IL-18 has demonstrated sensitivity to detect AKI in children undergoing cardiopulmonary bypass (CPB), increasing its levels between 4-8 hours after intervention (Parikh CR et al., 2006b). In the context of kidney transplant, IL-18 has shown increased levels 24 hours after intervention predicting delayed graft function (Parikh CR et al., 2006b). It can also differentiate between patients with established AKI from other kidney pathologies such as CKD or urinary tract infection (Parikh CR et al., 2004).

However, IL-18 value as AKI biomarker has been challenged in several studies because it does not increase its levels in adults which develop AKI after CPB or in contrast-induced nephropathy (Bulent Gul CB et al., 2008). Moreover, any inflammatory body response could result in an increase of IL-18 independently of AKI. Due to this data, IL-18 biomarker value is still controversial and needs further investigation (Vaidya VS and Bonventre JV, 2010).

Kidney Injury Molecule-1 (KIM-1):

KIM-1 is a type-1 transmembrane protein with an immunoglobulin and mucin domain. It is expressed in lymphocytes and proximal tubular cells in a low level, but its mRNA and protein drastically increase in response to kidney ischemic injury (Vaidya VS et al., 2006; Vaidya VS et al., 2009). This molecule is mainly expressed in regenerating proximal tubular cells that are dedifferentiated and proliferating (Sirota JC et al., 2011). After protein exposure in membrane, it is cleaved and the ectodomain is released into urine (Zhang Z et al., 2007).

KIM-1 levels are increased in patients with acute tubular necrosis (ATN) and it can be detected not only in urine samples, but also by immunohistochemical staining in tissue samples (Han WK et al., 2002). In the context of adult cardiac surgery, KIM-1 demonstrated low specificity and sensitivity values, but the diagnostic capacity of this biomarker increases when is combined with other molecules (Koyner JL et al., 2010). More prominent diagnostic potential was found in pediatric population submitted to CPB, where KIM-1 increases its levels 12 hours after surgery (Han WK et al., 2008). Urinary KIM-1 can also predict graft dysfunction and loss in kidney transplant context (Van Timmeren MM et al., 2007). Moreover, KIM-1 is expressed and can be detected by

immunohistochemistry in transplant biopsies with deterioration of kidney function and tubular injury, emerging as a possible new tool for pathologists (Zhang P et al., 2008).

Cystatin C:

Cystatin C is a 13 kDa protein of the cystatin family, produced by all nucleated cells (Westhuyzen J, 2006). Serum levels of this protein correlates with GFR better than serum creatinine and its values are not influenced by factors like muscle mass, protein intake, age or gender.

Cystatin C is freely filtered by the glomerulus and nearly completely reabsorbed by proximal tubules. Thus, increased levels of Cystatin C in urine reflect proximal tubule injury instead of glomerular dysfunction (Uchida K and Gotoh A, 2002; Conti M et al., 2006).

Cystatin C seems to be an early biomarker of AKI compared to serum creatinine (Herget-Rosenthal S et al., 2004). Indeed, several studies have demonstrated that urinary cystatin C can successfully predict AKI in the context of adult cardiac surgery (Koyner JL et al., 2008) and ICU patients (Nejat M et al., 2010). This biomarker can also predict delayed graft function in kidney transplant receptors and the urine cystatin C/Creatinine ratio during the first post-surgery days correlates with long-term graft function (Hall IE et al., 2011; Sirota JC et al., 2011). However, despite of this evidence, several works indicate that serum levels of cystatin C are a reliable marker of function, instead of a true biomarker for injury (Herget-Rosenthal S et al., 2004).

Despite the promising results observed for all these biomarkers, they still present several disadvantages that need to be solved to truly demonstrate their clinical value. One of the most significant problems is that, although some of them successfully predict AKI in pediatric population, they fail in adult studies, where other associated pathologies can be found. Due to these limitations, researchers are looking for new molecules with improved diagnostic potential, as we have done in this work.

3. Circulating microRNAs (miRNAs) as Novel Biomarkers:

miRNAs are small (20-25 nucleotides) endogenous RNA molecules which post-transcriptionally regulate gene expression and are involved in almost every cellular process (Krol J et al., 2010b). Despite of the previous hypothesis which postulated that miRNAs were only present inside the cell, it has been recently demonstrated that they can also be secreted to the extracellular environment with potential functional consequences (Février B and Raposo G, 2004). This secretion process makes them suitable to be detected in a wide range of cell-free body fluids such as breast milk, urine or serum (Fleischhacker M and Schmidt B, 2007).

miRNA secretion seems a highly regulated process and selection of miRNAs is a directed and non-random process. On the other hand, miRNA deregulation has been associated with the appearance and development of a wide range of pathologies. Due to these features, physiological or pathological regulation of intracellular miRNAs may also modify the panel of secreted miRNAs. Indeed, changes in serum miRNAs profiles have been unveiled as biomarkers of a wide range of diseases such as cancer, cardiovascular disease, stroke and multiple sclerosis, as well as altered physiological states such as pregnancy (for review Reid G et al., 2011).

Moreover, serum miRNAs have demonstrated great stability and resistance to aggressive conditions such as RNAse treatment, or drastic pH changes (Chen X et al., 2008). The mechanism underlying this unexpected miRNA stability in serum is not completely understood yet, but it has been demonstrated that circulating microRNAs are released from cells in membrane vesicles which protects them from the environment. Vesicles proposed to be carriers of circulating microRNAs include exosomes, which are 50-90 nm vesicles released by exocytosis from multivesicular bodies (MVB) (Février B and Raposo G, 2004; Kosaka N et al., 2010,) as well as larger microvesicles up to 1 µm (Hunter MP et al., 2008; Gallo A. et al., 2010). However, recent studies have demonstrated that serum circulating miRNAs can also be carried by Argonaute2 proteins (Arroyo JD. et al., 2011).

Taken together, all these features demonstrate that serum circulating miRNAs achieve nearly all the necessary characteristics for an ideal biomarker, as described before. Their presence in a peripheral fluid allows diagnosis by minimum invasive methods and serum samples can be easily and routinely obtained in clinical practice. In addition to their high stability in fresh serum, several studies have demonstrated that miRNAs maintains stability and reliability in long-term stored serum samples and in samples conserved at room temperature for hours (Chen X et al., 2008). Moreover, they can be easily quantified with relative low price and technical effort by qRT-PCR. These characteristics, join to

their great tissue and cell type specificity, have unveiled circulating miRNAs as promising biomarkers for more accurate diagnosis and monitoring of diseases.

4. – Renal Ischemia/Reperfusion Injury and Recovery:

Renal ischemia/Reperfusion (I/R) injury is one of the principal and more frequent causes of AKI (Liaño F and Pascual J, 1996). I/R is produced by a generalized or localized impairment of oxygen and nutrient supply to the kidney that can occur as a consequence of many pathophysiological conditions, like hemorrhage or sepsis, pharmacological treatments or clinical interventions, such as CPB and renal transplant (Bonventre JV and Yang L, 2011).

Although all kidney compartments are affected by I/R injury, proximal tubule epithelial cells are specially sensitive to lack of oxygen and nutrient supply due to their intrinsic characteristics (Gunaratnam L and Bonventre JV, 2009).

4.1. - Proximal tubule cells:

Proximal tubule cells are epithelial cells which present some metabolic and functional particularities due to their high specialization and the challenging environment where they are located:

1) *Cell Cohesion:* epithelial cells present a strong cellular adhesion network involved in epithelium stability, mechanotransduction, epithelial barrier impermeability and cell communication.

2) *Adhesion to basement membrane:* basement membrane is principally composed by collagen IV and glicoproteins and it is responsible for separating epithelial cells from connective tissue.

3) *Non-vascular tissue:* epithelia do not present blood vessels, thus its metabolism depends on oxygen and nutrient diffusion from connective tissue, located under basement membrane.

4) *Polarization:* proximal tubule cells are markedly polarized. They present an apical pole with a developed brush border to improve reabsorption of substances from urine. Basal pole is in contact with basement membrane and presents a great number of cell-matrix adhesion

structures. This marked polarity determines organelles distribution inside the cell and the diverse functions of plasma membrane in the different parts of the cell.

(For review Alberts B, 2007)

4.1.1. – Proximal Tubule Cell Adhesion Complexes:

As mentioned above, proximal tubule cells present two major types of cell adhesions: cell-to-cell and cell-to-extracellular matrix (ECM) (Figure 1A). Both structures are generally composed by a transmembrane cell adhesion molecule, intracellular scaffold or signaling proteins and cytoskeleton components.

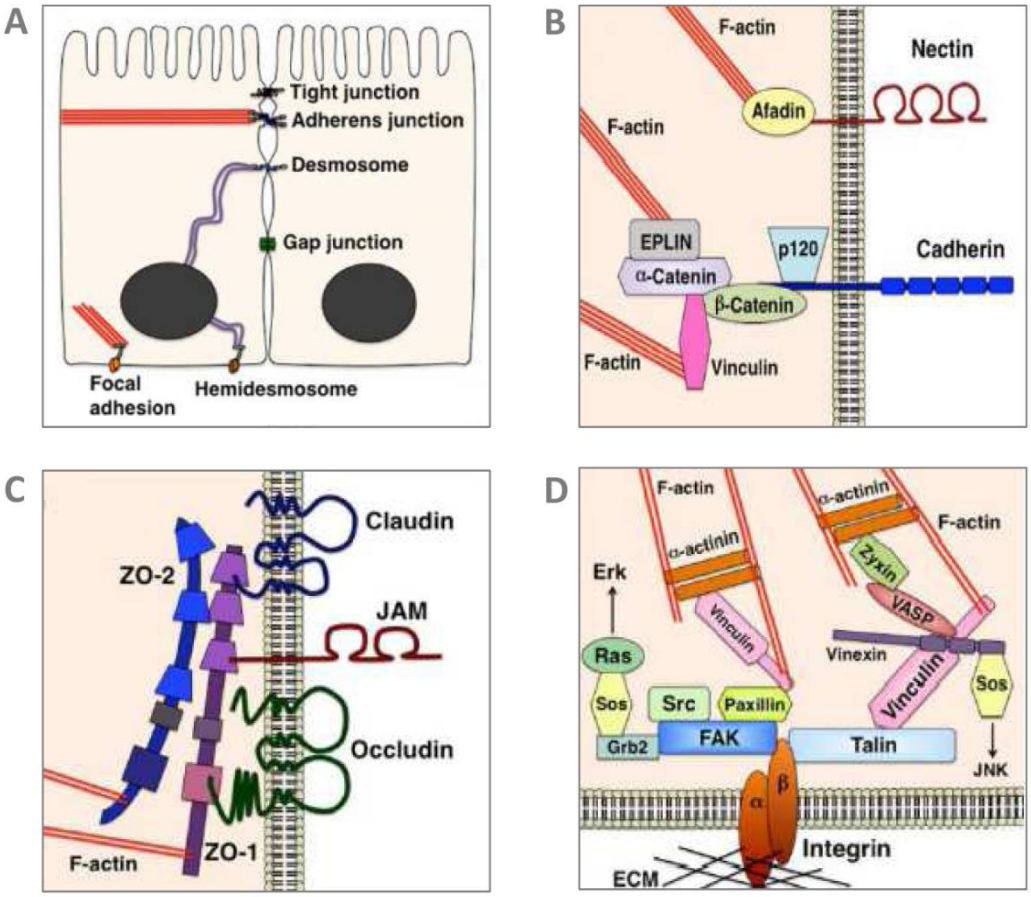

Figure 1: Epithelial cells adhesion structures. (A) Cell-Cell and Cell-Matrix adhesion structures location in epithelial cells (Red bars: actin filaments; Purple lines: intermediate filaments). (B) Molecular components of adherent junctions. (C) Molecular components of tight junctions. (D) Molecular components of focal adhesion complexes. Modified from Kawauchi T, 2012.

Proximal tubule cell-cell adhesion is mainly carried out by Adherent Junctions (AJ) and Thigh Junctions (TJ):

AJ are responsible of physical association of cells in a calcium-dependent manner. Cadherins are transmembrane proteins which are essential in these adhesive structures. Classical cadherins present an extracellular domain which provides trans-homophilic binding to other cadherins on neighboring cells, and their intracellular domains directly interact with β-catenin. α-catenin binds both β-catenin and several actin-binding proteins, such as vinculin and EPLIN. These actin-binding proteins regulate the interaction between cadherins and F-actin cooperatively with α- and β-catenins (Figure 1B) (Kawauchi T, 2012).

TJ are located at the most apical part of the lateral membranes in epithelial cells (Figure 1A). They are composed by a network of sealing strands formed by transmembrane proteins, mainly claudins and occludins, embedded in both plasma membranes. Both claudins and occludins bind to peripheral membrane proteins including intracellular PDZ domain-containing proteins, ZO-1. These peripheral proteins are responsible for anchoring TJ strands to the actin cytoskeleton (Figure 1C). In TJ strands, the intercellular distance between two neighboring cells is nearly zero, so that the tight junction restricts paracellular diffusion of substances and ions. In addition to the selective barrier function, tight junctions separate the plasma membrane into apical and basolateral domains to confine the diffusion of transmembrane proteins and lipids to the specific membrane domain as a fence (Denker BM and Sabath E, 2011; Kawauchi T, 2012).

On the other hand, proximal tubule cells are attached to basement membrane by focal adhesion complexes (FAC) (Figure 1A). These structures present a receptor molecule called integrin, which are transmembrane α–β heterodimers. Their extracellular domain can recognize ECM components such as fibronectin, laminin or collagen whereas their intracellular domain binds to many scaffold proteins including talin, vinculin, zyxin, focal adhesion kinase (FAK), Src or paxillin. These scaffolding proteins join FAC to the F-actin cytoskeleton and are involved in integrin activation and signal transduction. FAC not only present a cell-matrix adhesion function, but they can also act as a cell signaling center involved in the regulation of cell migration, morphological changes, survival and proliferation (Figure 1D) (Kawauchi T, 2012).

4.1.2 - Proximal Tubule Cell Trafficking:

Proximal tubule cells are responsible for reabsorption of a wide range of substances from glomerulus ultrafiltrate, determining urine composition and volume. These cells regulate body

fluid, ion and acid/base homeostasis trough the interaction of a great variety of channels, ion transporters and pumps within specific tubule segments, specific cells types and specific plasma membrane domains (Brown D et al., 2009). Due to this function, proximal tubule epithelial cells present a developed and complex intracellular trafficking activity which is essential for kidney function.

Proximal tubule cells are capable of endocytosis at either of their distinct apical or basolateral plasma membrane domains. Endocytosis occurs by both clathrin-dependent and –independent mechanism. The postendocytic fate (trafficking steps occurring after internalization) can vary. Briefly, the majority of the apically internalized fluid is thought to recycle to apical membrane or transcytose to basolateral membranes, but some is delivered to a complex population of late endosomes and lysosomes that also receive the majority of basolaterally internalized fluids (Apodaca G, 2001).

Apical endocytosis activity and trafficking is essential for receptor and transporters exposure and recycling in apical plasma membrane. Regulation of exposure of transporters plays a key role in proximal tubule cells reabsorption and, consequently, in kidney function regulation (Apodaca G, 2001).

4.1.3 - Proximal Tubule Cytoskeleton and motor proteins:

The cytoskeleton is crucial for the function of all eukaryotic cells and is required for mitosis, cytokinesis, cell motility, cell shape maintenance, endocytosis, and secretion. Epithelial cells exploit cytoskeletal elements to ensure efficient targeting of newly synthesized proteins from the trans -Golgi network to the appropriate cell surface domain. The cytoskeleton also plays a role in endocytosis, exit of cargo from early and late endosomes, and the transport, via transcytosis, of endocytosed proteins from one plasma membrane domain to the opposite (Apodaca G, 2001).

Proximal tubule cells present a developed cortical actin network which is mainly responsible for endocytosis and adhesion structures maintenance in the apical membrane. Actin remodelation seems to be a regulator of receptor-mediated endocytosis and formation of clathrin coated vesicles. On the other hand, actin cytoskeleton is also involved in transcytosis and protein delivery to late endosomes and lysosomes.

In proximal tubule cells, microtubules present a typical parallel organization with their minus ends toward the apical surface and their plus ends extending through the cell body to the basolateral surface (Hamm-Alvarez SF and Sheetz MP, 1998). Microtubules participate in

transcytosis and cargo delivery to endosomes and lysosomes (Apodaca G, 2001). Indeed, microtubule cytoskeleton is essential for organelle distribution and apical receptor recycling. Microtubule-based motor proteins Kinesin and Dinein play a central role in these distribution functions.

One of these motor proteins is Kinesin-2, a heterotrimeric, plus-end directed motor which comprises two motor subunits, KIF3A and either KIF3B or KIF3C, and a non-motor subunit KAP3. This complex is ubiquitously expressed and has been involved in the movement of Golgi/ER-Golgi intermediate compartment (ERGIC) membranes, Golgi-derived vesicles and late endosomes (Brown CL et al., 2005). Moreover, KIF3B, one of the motor subunits of kinesin-2, plays a key role in apical membrane receptor recycling in proximal tubule cells. This molecule is involved in the regulation of the exposure of kidney anion exchanger 1 (Duangtum N., et al 2011) and chloride/proton antiporter CLC-5 (Reed AA et al., 2010).

4.2. - Proximal Tubule Cell Response to I/R Injury:

As a result of this active intracellular trafficking, ion transporters activity and other ATP-dependent metabolic processes, proximal tubule cells have a very high oxygen demand, which make them especially sensitive to ischemic injury (Bonventre JV and Yang L, 2011).

As can be observed in Figure 2, sublethal ischemia results in rapid loss of cytoskeleton organization and cell polarity. The brush border quickly breaks down and the apical cortical cytoskeleton is disorganized, producing actin relocalization from apical to lateral plasma membrane (Sáenz-Morales D et al., 2006). Moreover, ATP depletion leads to intracellular free calcium concentration increase, which activates proteases and phospholipases that contribute to cytoskeleton disorganization and mitochondrial metabolism impairment (Bonventre JV, 2003).

ATP depletion and actin cytoskeleton reorganization also results in disruption of cell-cell and cell-matrix adhesion structures. TJ disruption compromises epithelial barrier function allowing glomerular filtrate backleak. Focal adhesion complexes are disassembled causing cell detachment from basement membrane and pro-apoptotic signaling (Bonventre JV, 2003; Bonventre JV and Yang L, 2011).

Detached epithelial cells and debris combine with proteins of the tubular lumen and fibronectin to produce casts that obstruct the tubules increasing I/R injury.

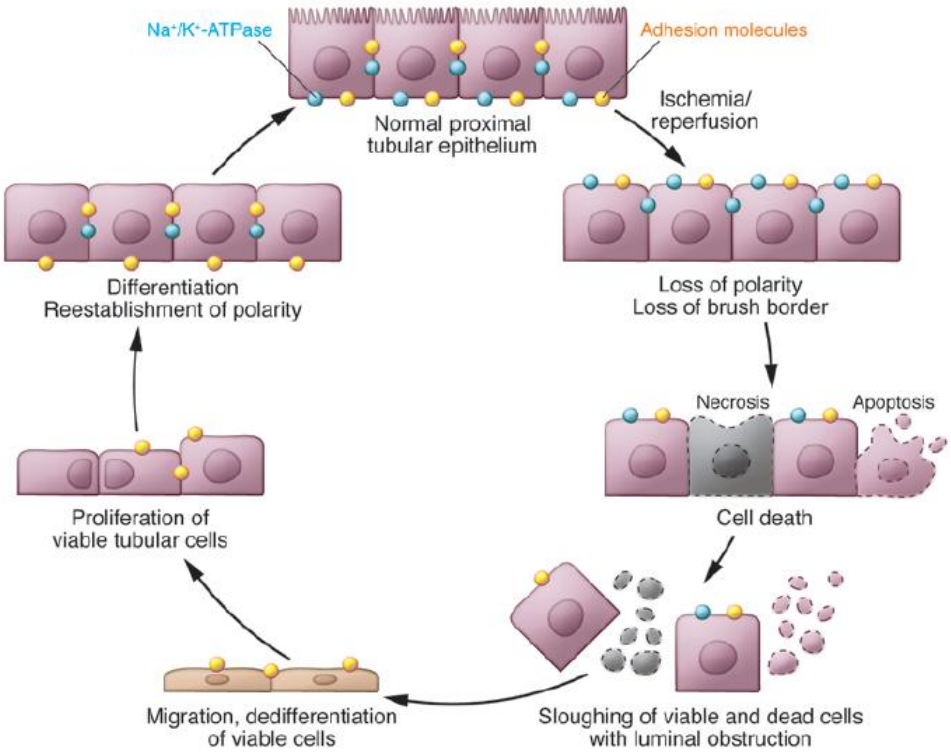

Figure 2: Proximal tubule cell response to I/R Injury: damage and repair process. Modified from Bonventre JV and Yang L, 2011.

In addition, loss of cell polarity, ATP depletion and cytoskeleton disorganization leads to incorrect targeting of membrane proteins such as ion transporters or endocytic receptors, producing proximal tubule function impairment (Bonventre JV and Yang L, 2011).

After injury, surviving proximal tubule cells suffer a dedifferentiation process and drastically increase their proliferation rate to refill the gaps produced by detached cells and promote tubule repair. Integrins delocalize from basal to lateral membrane contributing to viable cell migration along the basement membrane (Bonventre JV and Yang L, 2011). Proximal tubule cell replenishment has been a topic of intense debate in the last years. Some studies indicated that repair was carried out by bone marrow stromal cells or intrarrenal progenitors, but recent works have demonstrated that surviving epithelial cells are responsible for tubule recovery, although they need paracrine signals from bone-marrow derived cells (Duffield JS et al., 2005; Humphreys BD et al., 2008). After proliferation and migration, tubule cell number is restored and then epithelial cells differentiate, resulting in restoration of the functional integrity of the tubule and the nephron (Bonventre JV and Yang L, 2011).

5. – Regulators of Cell Responses to Oxygen: Hypoxia Inducible Factor.

Oxygen is a key factor that governs important cellular metabolism pathways in aerobic organisms. Because of this relevance, cells have developed adaptative and survival mechanism to maintain homeostasis in low oxygen tension conditions.

Hypoxia Inducible Factor (HIF) is the key regulator of cell responses to low oxygen conditions. HIF is a heterodimeric transcription factor composed by an α-subunit, which is continuously synthesized and degraded in the cytoplasm in normoxic conditions, and a β-subunit, which is non-oxygen sensitive and located in the nucleus (Rocha S, 2007).

Three isoforms have been identified for the α-subunit (HIF-1α, HIF-2α and HIF-3α). HIF-1α and HIF-2α isoforms have been extensively studied in the last years. Although there are some coincidences and overlapping, both subunits present non-redundant activities driving the expression of different sets of genes and being preferentially expressed in different cell types (Rocha S, 2007; Heyman SN et al., 2011).However, the role and functions of HIF-3α subunit still remains unclear. In this regard, some studies postulate a dominant-negative function of HIF transcriptional activity (Heyman SN et al., 2011). Two isoforms have been identified for β-subunit, but their biology and functions have not been elucidated yet (Brahimi-Horn MC and Pouysségur J, 2009).

Under normoxic conditions α-subunits are constantly synthesized but not accumulated since they are rapidly hydroxylated by oxygen-dependent prolyl-4 hydroxylases domain enzymes (PHD). Hydroxylated α-subunits are recognized by Von-Hippel-Lindau protein (VHL), a component of E3 ubiquitin ligase complex, which covalently links a chain of ubiquitin residues to α-subunits, targeting them to be degraded by the proteasome.

Under low oxygen conditions, PHD activity is reduced and α-subunits accumulate in the cytoplasm. When stable, α-subunits bind to nuclear pore proteins and translocate into the nucleus to form αβ-heterodimers. These dimers recognize and bind to DNA sequences known as Hypoxia Response Elements (HRE) located in the promoter or enhancer region of target genes, whose expression is activated (Rocha S, 2007; Heyman SN et al., 2011). To activate the majority of its target genes, HIF needs to associate with the transcriptional coactivators p300 or CBP. However this binding is not necessary for all HIF target genes, indicating that other transactivating factors or mechanisms are possible (Rocha S, 2007).

There are more than 100 HIF target genes identified in humans. They participate in key pathways in cell metabolism and survival, including cell functions necessary for hypoxia adaptation such as

erythropoiesis (EPO), increased glucose intake (Glucose transporter-1), metabolism switch to glycosysis (several glycolysis enzymes), lactate metabolism (Lactate dehydrogenase), angiogenesis (VEGF), vasodilatation (Inducible Nitric oxide synthase) and free radicals scavenging (HO-1). Moreover, there is an increasing evidence of genes whose expression can be repressed by HIF (Heyman SN et al., 2011). Recently several miRNAs have been described as HIF targets, including miR-210 (Rocha S, 2007; Chan YC et al., 2012).

5.1. – Role of Hypoxia Inducible Factor (HIF) in Renal Ischemia/Reperfusion Injury:

As a key regulator of cells responses to low oxygen tensions, HIF is also an essential transcription factor in the renal response to ischemia. In the last years, several works have evidenced the critical protective role of HIF expression against renal I/R.

Ischemic preconditioning, produced by a transient and mild I/R stimulus, confers kidney protection against a subsequent acute ischemic injury by activating several cell signaling pathways, including HIF-1α and HIF-2α. Moreover, accumulation of HIF-1α and HIF-2α by pharmacological inhibition of PHDs or DMOG administration to mice results in renal protection against ischemia. On the other hand, HIF-1α and HIF-2α heterozygous knockdown (KO) mice are more susceptible to I/R injury. (Hill P et al., 2008).

In another experimental approach, conditional inactivation of VHL protein in mice and subsequent HIF accumulation resulted in tolerance to renal I/R (Iguchi M et al., 2008). In an allogenic model of renal transplant in rats, donor pre-treatment with PHD inhibitor prevented graft injury and increased receptor survival (Bernhardt WM et al., 2009).

As previously mentioned, different α-subunits present non-redundant activities and specific expression patterns in each organ and cell type. This is the case of the kidney, in which HIF-2α is mainly expressed in erythropoietin producing interstitial fibroblast and peritubular endothelial cells, whereas HIF-1α is mainly expressed in tubular epithelial cells (Rosenberg C et al., 2002).

Consequently, HIF-1α is the main controller of I/R response in proximal tubule cells. Moreover, recent studies have pointed out that HIF-1α is essential for proximal tubule cell survival during ischemia but also in reperfusion. HIF-1α KO by siRNA administration *in vivo* aggravates renal I/R injury and exacerbates proximal tubule damage (Conde E et al., 2012). In addition, HIF interference during reperfusion leads to proliferation/cell death imbalance and impaired tubule repair (Conde et al., *in preparation*).

6. – microRNAs:

miRNAs are small (20-25 nucleotides) non-coding RNAs that have revolutionized our understanding of gene post-transcriptional regulation. They are fine tuners of gene expression and more than 50% of the genes in mammals are submitted to their control, participating in almost every cellular function. Moreover, their deregulation is frequently associated with disease appearance and development.

There have been described approximately 800 miRNAs in humans, a number comparable to the known number of transcription factors or other regulatory proteins. Moreover miRNAs show a very specific expression patterns that are different among tissues and cell types.

Their mechanism of action is based on recognition of small sequences in their target mRNAs. This feature make possible that one miRNA can regulate hundred of target mRNAs and that one mRNA can be regulated by several miRNAs. This dynamic regulation has unveiled them as key regulators of a wide range of cellular events, including rapid responses to stress (Krol J et al., 2010b).

6.1. – microRNA Biogenesis:

miRNAs are mainly transcribed by RNA polymerase II as longer primary transcripts called pri-miRNAs. miRNAs genes are often located in non coding DNA regions and they are frequently organized in clusters. Clustered miRNAs are transcribed as a single, longer pri-miRNA which generates several functional miRNAs by subsequent processing. miRNAs genes can also be found in protein coding genes, specially located in introns. In these cases, splicing of the coding mRNAs leads to generation of the functional miRNA. As other transcripts produced by RNApolymerase II, pri-miRNAs presents 5´Cap and 3´Poly-A Tail.

Pri-miRNAs molecules form imperfect stem-loop structures that can be recognized by a processing complex formed by the RNAse III enzyme Drosha and the RNA binding protein (RBP) DGCR8. Stem-loop double stranded structures of pri-miRNAs are recognized by DGCR8, which guides de positioning of Drosha. This catalytic center cleaves pri-miRNAs liberating a hairpin RNA molecule of 70-100 nucleotides known as pre-miRNA.

Pre-miRNAs are exported to the cytoplasm by the nuclear export receptor Exportin 5 in a Ran-GTP dependent manner. In the cytoplasm pre-miRNAs are further processed by other RNAse III enzyme called Dicer. A new cleavage produces a double stranded RNA molecule of 22 nucleotides. One of the

strands (the mature miRNA) is transferred to the Argonaute protein to conform the RNA Induced Silencing Complex (RISC). The other strand (often called minor, passenger or *strand) is frequently degraded. Strand selection mechanism has not been completely elucidated yet. However, it has been proposed that the strand with the less stable base-pairing in its 5´end is often chosen as guide strand to be loaded in the RISC complex.

RISC complex is the key effector of miRNA regulation. This complex, joined to other controlling factors, is responsible for driving mRNA degradation or translation repression, as will be further detailed in following sections (For review Krol J et al., 2010; Treiber T et al., 2012).

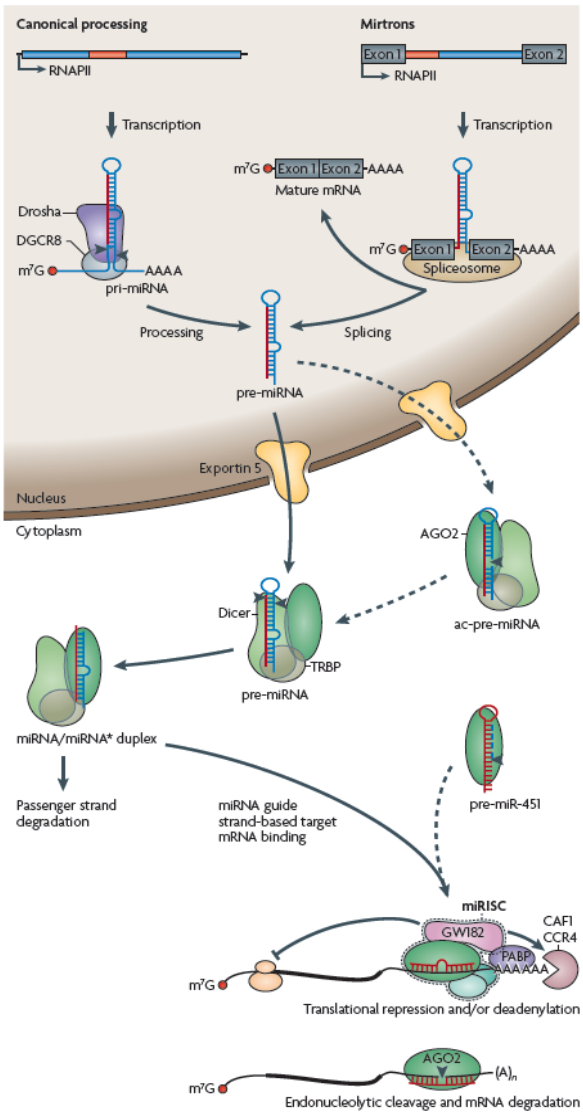

Figure 3: MicroRNA biogenesis and function. Modified from Krol J et al., 2010b.

6.2. – miRNA Biogenesis Regulation:

Given the great importance of miRNAs in gene expression regulation and cell function, miRNA biogenesis is also submitted to a tight regulation.

The first control step corresponds to pri-miRNA transcription. Intronic miRNA expression is usually governed by the promoter of the host gene. However approximately one third of the intronic miRNAs presents additional promoters that allow independent regulation. Intergenic miRNA generally present their own promoters, which show all the characteristics of RNA polymerase II transcription, such as CpG islands. Clustered miRNAs usually share one promoter for all the encoded miRNAs and are transcribed as part of long pri-miRNAs (Treiber T et al., 2012).

In addition to transcription regulation, pri-miRNA processing can be controlled in RNAse III cleavage steps, leading to the accumulation of different intermediate precursors.

Drosha processing can be regulated by growth factors such as Bone Morphogenic Protein (BMP) or Tumor Growth Factor-β (TGF-β), which can stimulate the processing of specific miRNAs (Davis BN et al., 2010). Moreover DNA damage-induced p53 pathway can enhance the processing of miRNA implicated in tumor suppression functions (Suzuki Hi et al., 2009). In addition, RNA binding proteins involved in mRNA splicing and editing can also regulate this step (Trabuchi M et al., 2009; Yang W et al., 2006).

Dicer processing is regulated by Lin-28 in embryonic pluripotent cells. This protein binds to the terminal loop of Let-7 family pre-miRNA preventing Dicer cleavage. Lin-28 protein levels are high in embryonic stem cells producing repression of let-7 family miRNAs and overexpression of their targets, which are involved in pluripotent state maintenance (Viswanathan SR et al., 2008).

6.3. – miRNA Target Recognition and Function:

Once loaded into the RISC complex, miRNAs recognize their target mRNAs by base-pair complementarity. Target sequences are mainly located in the 3´ untranslated region (UTR) of mRNAs. However, functional miRNA binding sites can be also found in the 5´ UTR and open reading frame regions.

Nucleotides in positions 2-8, called seed sequence, are essential for pairing with the target mRNA and miRNA function. In the case of perfect complementarity of the seed sequence of the miRNA with

the target sequence, miRNAs act as a short interfering RNA (siRNA) promoting mRNA cleaving by RISC complex. This mechanism is principally found in plants but is very rare in mammals.

When pairing with target sequences is partially complementary, which is the most frequent mechanism in mammals, miRNA regulation is produced by mRNA translation repression or degradation. However this degradation process is different and involves recruitment of deadenylase complexes that remove or shorten the poly-A tail of the target transcript. Poly-A tail shortening induces decapping of the 5´ extreme of the transcript and uncapped mRNAs are rapidly degraded by 5´ to 3´ exoribonucleases(Figure 4) (For review Krol J et al., 2010b; Treiber T et al., 2012).

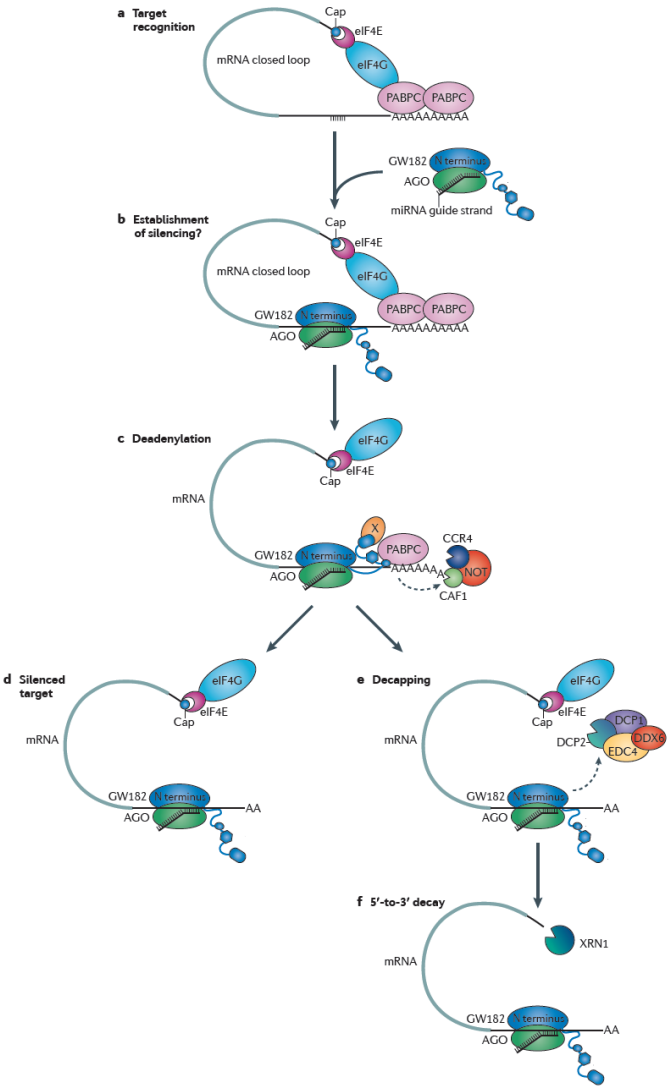

Figure 4: Mechanisms of miRNA-mediated silencing in animals. Modified from Huntzinger E and Izaurralde E, 2011.

Translational repression has been proposed to occur in four different ways:

1) Inhibition of translation initiation.
2) Inhibition of translation elongation.
3) Co-translational protein degradation.
4) Premature termination of translation.

For inhibition of translation initiation increasing evidence suggest that animal miRNAs interfere with the function of the eukaryotic translation-initiation complex 4F (eIF4F) and Cytoplasmic poly-A Binding Protein (PABPC).

Post-initiation stage regulation hypothesis are evidenced based on a common observation: miRNAs and their targets were associated with polysomes, isolated by sucrose sedimentation gradients. These polysomes were sensitive to conditions that inhibit translation, so they were considered to be active.

Several works demonstrated that target mRNAs were translated but no corresponding protein was found, probably due to a co-translational degradation of the nascent polypeptide. Other model hypothesizes that miRNAs may cause premature dissociation of ribosomes during translation (ribosome drop off). Moreover, recent evidence indicates that miRNAs can regulate translation independently of iniciation factors through internal ribosome entry sites (IRES) (for review Huntzinger E and Izaurralde E, 2011).

Although contribution rate of mRNA decay and translational repression to miRNA action is a controversial topic, it seems clear that target degradation provides a major contribution to silencing in mammal cells. In this regard, it has been estimated that mRNA decay is present in 85% of miRNA regulation process whereas 15% corresponds to translational repression (Treiber T et al., 2012; Huntzinger E and Izaurralde E, 2011).

Despite these recent advantages, miRNA target recognition and function mechanisms are not completely understood yet. In the last years many bioinformatics approaches have been developed to facilitate miRNAs target prediction and *in silico* functional studies. Several target prediction programs can be currently found online, such as microcosm (Griffiths-Jones S. 2008) (www.ebi.ac.uk/enright-srv/microcosm), Targetscan 4.1 (Friedman RC. et al., 2009) (www.targetscan.org/vert_40/) and Pictar I (Krek A. et al., 2005) (www.pictar.mdc-berlin.de). These prediction tools principally base their algorithms in sequence complementarity between the 5´seed region of the miRNA and the 3´UTR of the target gene.

6.4. – Regulation of miRNA function:

miRNA pathway is strictly controlled not only during miRNA biogenesis but also during miRNA function, where several regulation steps can be found.

In many organisms there are several AGO proteins operating in miRNAs pathways. Recent evidence suggest that AGO isoform selection is a step in miRNA function regulation. Mammal cells present 4 AGO proteins. Although AGO2 is the only one which is able to cleave perfect complementary RNA targets, the four isoforms present overlapping functions with weak miRNA sorting preference. However, they seem to differ in their ability to repress protein synthesis. Thus differences in AGO relative abundance may affect the strength of miRNA repression in particular tissues or cell types. Moreover, as different miRNAs could be preferentially loaded in different AGO isoforms, changes in cell concentration of individual AGO proteins can also modify the range of miRNAs associated to RISC complex.

Cytoplasmic compartmentalization can control access to binding proteins or concentrate factors which participate in the same cellular pathway to facilitate a determinate cell function. In this regard, compartmentalization structures such as processing bodies (P-Bodies) and multivesicular bodies (MVB) have emerged as potentially relevant elements for miRNA function.

Translationally repressed mRNAs can accumulate in cytoplasmic structures called P-Bodies. These complexes participate in mRNA storage and decay and, consequently, they are enriched in proteins involved in translational repression and mRNA deadenylation, decapping and degradation. Several studies have demonstrated that RISC complex containing miRNAs and their corresponding target genes are located in P-bodies, clearly linking these structures with miRNA function. However, it is important to notice that it P-bodies formation could be also a consequence of miRNA-mediated repression rather than an essential element for miRNA function.

MVB have been identified as contributors to miRNA function and secretion. In their lumen, MVB accumulate vesicles that can be secreted or delivered to lysosomes for degradation. Blockade of MVB formation inhibits miRNA silencing whereas blocking of MVB turnover stimulates miRNA target repression. Moreover, MVB formation blockade leads to impaired small RNA loading in RISC complexes, evidencing that MVB play a key role in RISC turnover.

(For review Krol J et al., 2010b)

Selective loading of RISC complex proteins and microRNAs into MVB vesicles to be released as exosomes constitutes an essential step in miRNA secretion regulation. This step is essential for miRNA function as biomarkers in body fluids, as indicated in previous sections of this work.

6.5. – miRNA decay and turnover:

Accumulative evidence reveals that different miRNAs present distinct and inherent half-lives that could be encoded by their sequence or can be determined after maturation by post-transcriptional mechanisms such as uracile and adenosine addition to their 3´end.

Half-lives of some miRNAs could reach many hours or even days in some organs like the liver or the heart (van Rooij E et al., 2007; Gatfield D et al., 2009). However, their ability to regulate rapid cell responses to environmental signals makes this slow turnover rate not appropriate for every context. Indeed, some miRNAs expressed in retina and involved in darkness adaptation presents half-life of approximately 1 hour (Krol J et al., 2010a).

These interesting data points out that more attention needs to be focused in miRNA turnover because miRNA half-life regulation could emerge as potential critical step in miRNA function.

7. – microRNAs in Kidney:

Due to the relevance of miRNA function and their determinant participation in a wide range of processes such as organ development, homeostasis and pathophysiology, miRNA studies in kidney development and function has emerged as a field of intense research during the last few years.

Lack of miRNA activity causes defects in kidney terminal differentiation such as decreased proliferation rate, aberrant nephron patterning and delayed terminal differentiation of kidney tubules. miR-30 and miR-200 family are two of the most expressed miRNAs in the kidney. Their role is essential during kidney development as they strictly regulate the temporal and spatial expression pattern of transcription factors involved in pronephron maturation (Wessely O et al., 2010).

In addition, the expression pattern of several miRNAs changes as kidney development progresses, activating or silencing their expression when renal epithelial cells are terminally differentiated and cell-fate determination has concluded.

Studies based on DICER knockdown in podocytes, juxtaglomerular cells and proximal tubules have unveiled the role of miRNAs in kidney function maintenance as well as specific miRNA roles in each kidney compartment.

KO of DICER in glomerulus results in progressive loss of podocyte function leading to proteinuria and kidney function impairment and ultimately leading to death (Shi S et al., 2008; Ho J et al., 2008). Moreover, DICER deletion in juxtaglomerular cells produces acute loss of this cell type leading to an abrupt decrease of renin expression in kidney (Sequeira-Lopez ML et al., 2010).

All these studies indicate that miRNAs play critical roles in normal renal function and physiology maintenance and when altered, may lead to renal diseases. Specific miRNAs deregulation has been linked to renal disease appearance and development. For instance, miR-192 has been identified as a critical regulator of collagen production in diabetic nephropathy (Kato M et al., 2007).

The clear role of miRNA in kidney function and disease and the ability of miRNAs to regulate rapid responses to stress and injury, have unveiled microRNAs as potential key regulator of kidney responses to acute damage, such as ischemia/Reperfusion injury.

7.1. – miRNAs in Renal Ischemia/Reperfusion:

Despite of the intense research in miRNAs in kidney during the last years, publications about microRNAs in renal I/R injury are still scarce. However, miRNAs involvement in kidney response to I/R was undoubtedly demonstrated by DICER KO experiments in proximal tubular cells. DICER KO mice showed normal renal function and proximal tubule structure in basal conditions. However, general downregulation of miRNAs conferred a very high protection against I/R reperfusion injury with significantly better renal function, less tissue damage, lower tubular apoptosis and improved survival compared to wild-type animals (Wei Q et al., 2010).

Global expression profiling experiments in mice which underwent renal I/R have identified a signature of 9 microRNAs (miR-21, miR-20a, miR-146a, miR-199a-3p, miR-214, miR-192, miR-187, miR-805, and miR-194) which are differentially expressed in kidney compared to sham controls (Godwin JG et al., 2009). Moreover, a more recent work from the same group demonstrates that microRNA signature after I/R is different in operated animals compared to sham controls and these differences maintain and evolve along time. Their data strongly evidence that differential expression of miRNAs after I/R constitute a new biomarker for renal I/R injury (Shapiro MD et al., 2011).

A recent study of Liu F. et al. demonstrates that miR-210, a well established miRNA regulated by hypoxia, is involved in angiogenesis regulation during renal I/R by activating VEGF and its receptor VEGFR2 expression (Liu F. et al., 2012).

In addition, miRNAs contained in vesicles derived from endothelial progenitors protects renal cells from I/R injury. Intravenous injection of microvesicles conferred functional and morphologic protection of renal cells by enhancing tubular cell proliferation and reducing apoptosis and leukocyte infiltration. These miRNAs also protect kidney from chronic damage progression by inhibiting glomerulosclerosis and tubulointestitial fibrosis. Thus, vesicle-derived miRNAs contribute to reprogramme resident renal cells to a regenerative program after I/R injury (Cantaluppi V. et al., 2012).

The improved knowledge of miRNAs involved in renal I/R injury and recovery could be a key point to develop new therapeutic approaches to prevent AKI, reduce chronic diseases derived from acute episodes and improve patient outcome. Moreover, identification of microRNAs as novel biomarkers for AKI could drastically change current clinical practice allowing earlier detection and better patient monitoring and management.

HYPOTHESIS

AKI is a clinical syndrome characterized by very high mortality rates and hospitalization costs, becoming a serious health problem in developed countries. Recent evidence have demonstrated that AKI is a complex syndrome which includes both structural and functional kidney injury and whose underlying cellular and molecular mechanism are not well understood yet. Ischemic damage is one of the most frequent causes of kidney injury and it is present in several clinical contexts such as cardiac surgery or renal transplant. I/R injury mainly affect proximal tubule cells provoking cytoskeleton and adhesion structures alterations and proximal tubule function impairment. Identification of molecular mechanisms underlying kidney ischemic injury and recovery is essential for improving our understanding of AKI pathophysiology and identification of new therapeutic targets.

In addition, current clinical tools for AKI detection and management present important limitations which delay AKI diagnosis and treatment with dramatic consequences in patient outcome. Novel biomarkers are needed for earlier diagnosis, detect patients at risk and obtain information about AKI etiologies and origin. Moreover, these novel biomarkers should be measured in patient samples obtained by minimum invasive methods and detected by cost-effective techniques.

microRNAs are small endogenous RNA molecules which regulate almost every cellular process by post-transcriptional mechanisms. They are mediators of long-term processes such as development and differentiation, but their dynamic regulation has unveiled them as key mediators of rapid cell responses to environmental stimuli and stress, such as ischemia. Hundred of miRNAs have been described but these molecules present a precise and highly regulated expression pattern with tissue and cell type specific expression profiles.

Moreover, in the last five years, accumulative evidence has demonstrated that miRNAs not only exert their function intracellularly, but they can also be secreted to the extracellular environment. This secretion pathway makes possible the detection of miRNAs in extracellular body fluids such as serum, where they have demonstrated a great stability. Moreover, changes in serum microRNA profiles have been associated to pathologies such as cancer or physiological states such as pregnancy. These characteristics have unveiled miRNAs as potential biomarkers for pathology diagnosis and monitoring.

Based on these previous premises, we proposed that miRNAs and their target genes could be key regulators of kidney responses to injury, including I/R damage and recovery. Moreover, due to their great cell and tissue specificity, secreted extracellular miRNAs detected in serum could be novel and accurate biomarkers of AKI which could contribute to a more precise diagnosis, prognosis and management of AKI patients.

OBJECTIVES

The principal objective of this work is the study of miRNAs involved in the kidney response to I/R injury and their putative role as AKI biomarkers detected in human serum samples.

To reach this general aim, we propose the following partial objectives:

1. Identification of miRNAs regulated in response to I/R injury using an *in vitro* model of Hypoxia/Reoxygenation in proximal tubule cells and an *in vivo* model of renal Ischemia/Reperfusion in rat.

2. Characterization of mechanisms responsible for miRNA expression modulation, including HIF-1α.

3. Study of the biological significance of identified miRNAs in proximal tubule cells submitted to H/R.

4. Identification of a serum miRNA profile for AKI diagnosis in human samples.

5. Validation of selected serum miRNAs in a cohort of ICU patients compared to healthy controls and in a cohort of patients undergoing cardiac surgery with CPB.

6. Study of diagnostic and prognostic values of identified miRNAs, establishing appropriated statistical correlations with clinical data.

MATERIAL AND METHODS

1.- Cell culture and Hypoxia/Reoxygenation (H/R) Protocol:

NRK-52E cells (ATCC) were cultured in DMEM containing 10% FBS, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen). HK-2 cells (ATCC) were cultured in DMEM/F12 containing 10% FBS, 1 g/l insulin, 0.55 g/l transferrin, 0.67 mg/l selenium, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin (Invitrogen). Both cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 ºC.

For H/R protocol, cells were grown until confluence and then they were serum deprived for 24 hours. For minimum medium hypoxia (HMM), monolayers were cultured for 6 hours in HBSS (Invitrogen), in a low oxygen atmosphere containing 1% O_2, 94% N_2, 5% CO_2 (Air Liquide). For reoxygenation, complete medium was added and plates were placed in a regular incubator with 21% O_2 (Sáenz-Morales D et al., 2006). In complete medium hypoxia (HCM), cells were serum starved as described above and then submitted to hypoxic atmosphere for 6 hours. Normoxic cells (Nx) were serum deprived without nutrient and oxygen tension changes. Serum-starved cells following 6 h in HBSS correspond to nutrient depletion control condition (CC) (Figure 5A).

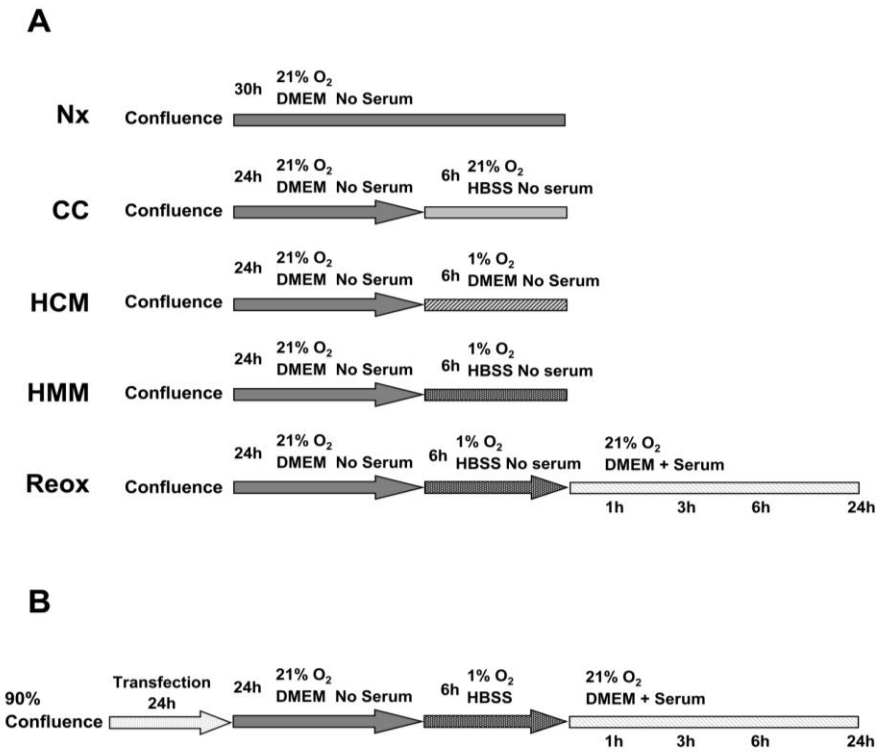

Figure 5: Hypoxia/Reoxygenation and transfection protocols. (A) Schematic representation of H/R protocol where changes in nutrients and oxygen tension are indicated for each condition, represented in different arrows. (B) Transfection and H/R protocol diagram. Cells are transfected with HIF-1α siRNA, in the case of HK-2 cells, or pre/anti-miR-127 in NRK-52E cells. 24 hours after transfection, cell cultures undergo H/R protocol as described above.

Deferoxamine is an iron chelator usually used as hypoxia-mimetic compound to stabilize HIF-1α through PHDs inhibition. Deferoxamine is added to culture medium to a final concentration of 300 μM for 8 hours.

2.- Renal Ischemia/Reperfusion model in Rat:

Experimental procedures were performed according to the European Community guidelines (EC609), Spanish laws (RD 1210/2005) and approved by the Internal Committee for Animal Ethics of Hospital Universitario Ramón y Cajal. Male Sprague Dawley rats (180–200 g) were anesthetized with an inhaled anaesthesia mixture of 2% isoflurane (Abbott Laboratories Ltd.) and 1 l/min oxygen. Renal I/R injury was performed after laparotomy by a 45 min bilateral clamping of renal pedicles. Sham operated animals underwent the same surgical procedure without clamping. Animals were sacrificed at 0, 24 h, and at 3, 5 and 7 days after reperfusion.

Blood samples were obtained before sacrifice by punction of the Cava vein. For serum extraction tubes were incubated at room temperature for 30 min and then centrifugued at 2500 rpm for 10 minutes at 4ºC. Kidneys were obtained by dissection of renal pedicles. Half of each kidney was directly frozen liquid nitrogen for protein and RNA extraction and the other half was fixed in formalin and paraffin embedded for further analysis.

3.- HIF-1α siRNA transfection *in vitro:*

HK2 cells at 80-90% of confluence were transfected with 100 nM HIF-1α siRNA (sc-44225, Santa Cruz Biotechnologies) or scramble siRNA (sc-37007, Santa Cruz Biotechnologies), using Lipofectamine 2000 (Invitrogen, Barcelona, Spain) according to manufacturer's protocol. Transfection is carried out for 5 hours and then stopped by adding complete medium to plates. Transfected cells were submitted to H/R after 24h of transfection (Figure 5B). Samples were harvested at appropriate times in each experiment.

4.- Pre-miR and anti-miR transfection *in vitro:*

NRK-52E cells at 80-90% confluence were transfected with 100nM anti-miR-127 (AM17000, Anti-miR™ miRNA Inhibitor, Life Technologies) y anti-miR-Scramble (Anti-miR™ miRNA Inhibitors-negative Control #1, Life Technologies) using 1.5μl of lipofectamine 2000 in a 24 well plate. Pre-miR-

127 (AM17100, Pre-miR™ miRNA Precursor Molecule, Life Technologies) and Pre-miR-Scramble (AM17110 Pre-miR™ miRNA Precursor Molecules—Negative Control #1, Life Technologies) were transfected with a final concentration of 0.1 nM using 0.5 μl of lipofectamine 2000 per well. Pre-miR and anti-miR final concentrations were chosen after setting experiments, checking miR-127 expression by qRT-PCR. Treated cells were submitted to H/R protocol after 24 hours of transfection (Figure 5B).

5.- Tissue and cell culture RNA extraction and Real-Time PCR:

Total RNA was isolated from renal tissue and cell cultures with TriPure Isolation Reagent (11667165001, Roche Diagnostics) by mechanical lysis and Phenol/Chloroform extraction. Rat kidney cortexes were previously manually dissected containing a high proportion of proximal tubules. RNA concentration and purity was measured with Nanodrop device (Thermo-Fisher) and RNA integrity was checked by agarose gel analysis.

2 μg of RNA were used to obtain cDNA using Transcriptor First Strand cDNA Synthesis Kit (04897030001, Roche Diagnostics). 1μl of cDNA was used as template for quantitative PCR reaction with SYBR Green (11066420, SYBR Green I Master, Roche Diagnostics). Ribosomal 28s gene was used as housekeeping for data normalization. Primers employed were: *28s*, forward: CAGTACGAATACAGACCG, reverse: GGCAACAACACATCATCAG; *Rat KIF3B*, forward: ATCATACAAACGAGCAGCAG, reverse: GTCTCTTTCAGTTCCAAGGTC; *Human KIF3B*, forward: GCCATTGTAGAGGATCACAG, reverse: CAACAAGCAACTTACTCTCCA.

All reactions were carried out in a Light Cycler 480 equipment (Roche) with the following temperature protocol: 40 cycles of 95 ºC 10 sec., 60ºC 20 sec. and 72 ºC 20 sec. Quantification crossing points (Cq) were calculated with 2^{nd} derivative method (Light Cycler 480 Software 1.5, Roche) and data analysis was performed using $2^{-\Delta\Delta Cq}$ formula.

6.- microRNA quantification by Taqman Assays:

Commercially available taqman microRNA assays (Life Technnologies) were used for miRNA quantification by real-time PCR in rat kidney tissue and cell cultures, after extraction and quantificacion of RNA as previously described.

50 ng of total RNA was used for retrotranscription reaction using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems). PCR was performed using TaqMan microRNA Assays (Applied Biosystems) following manufacturer´s instructions with the following temperature protocol: enzyme activation at 95ºC for 10 min.; 40 cycles of 95 ºC 15 sec. and 60ºC for 60 sec. RNU6B was used as internal control for data normalization in cell culture studies, whereas ribosomic RNA 5s was employed for rat kidney tissue.

All reactions were carried out in a Light Cycler 480 equipment (Roche). Quantification crossing points (Cq) were calculated with 2^{nd} derivative method (Light Cycler 480 Software 1.5, Roche) and data analysis was performed using $2^{-\Delta\Delta Cq}$ formula.

7.- Protein extraction and Western Blot analysis:

Cell cultures were harvested in lysis buffer (0.25 M Tris pH=6.8, 6% SDS, 10% glycerol, 20 mM DTT, Bromophenol Blue, protease and phosphatase inhibitors) (Sigma-Aldrich). Homogenates were mechanically disrupted by syringing and then centrifugued. Precleared supernatants were resolved by SDS-PAGE and transferred to nitrocellulose (Hybond-ECL Amersham, GE Healthcare). Primary antibodies used: anti-human anti-HIF-1α (90-110 KDa), 1:250 (BD Transduction Laboratories); anti-KIF3B (85KDa) 1:500 (SC-50456, Santa Cruz Biotechnologies); anti-β-actin (42KDa) 1:1000 (Sigma-Aldrich). Appropriate horseradish peroxidase-conjugated secondary antibodies (Dako) were used. Specific signals were visualized using enhanced chemiluminescence (Amersham Biosciences).

8.- Immunofluorescence:

NRK-52E cells were grown on coverslips coated with collagen IV (1 μg/mL. Sigma-Aldrich). To visualize actin cytoskeleton, cells were fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked in PBS 1% BSA and stained with Phalloidin-Alexa568 1:40 (Invitrogen), 30 min at room temperature. For paxillin and ZO-1 inmunostaining, cells were processed as described above and incubated with PBS 1% BSA containing primary antibodies anti-paxillin (Millipore) 1:250 and anti-ZO-1 (Invitrogen) 1:200, 1 hour at room temperature. Appropriate secondary antibodies anti-mouse Alexa488 or anti-rabbit Alexa488 (Invitrogen) were used 1:250 for 1 h at room temperature. After washing, samples were mounted with prolong gold antifade reagent with DAPI (Molecular probes). Images were obtained with Spectral Confocal Microscope TCS SP5 (Leica Microsystems).

9.- Non receptor-mediated endocytosis assay:

Pinocytosis capacity of proximal tubule cells was estimated by internalization of fluorescent dextran. Dextran-FITC of 70 KDa (SD70S, Sigma-Aldrich) to a final concentration of 1 mg/ml was added to NRK-52E culture medium 6 hours before sample collection. After incubation, samples were fixed in 4% paraformaldehyde and coverslips were mounted using prolong antifade reagent (Invitrogen) with DAPI. Images were obtained with Spectral Confocal Microscope Leica TCS SP5.

Quantification of pinocytosis was performed using NIS-Elements BR Image Software (Nikon). For each image, surface of DAPI signal and green signal was estimated in pixels^2 and quantification was expressed as a Green/DAPI surface ratio.

10.- Identification of Putative HRE Elements:

For the identification of HIF binding sites within the mir-127 locus we follow the strategy described elsewhere (Ortiz-Barahona A et al., 2010). Briefly, we first identified mammal or vertebrate PhastCons elements (Siepel A et al., 2005) within the region chr14:100418481- 100419663 containing the mir-127 gene. Adjacent PhastCons elements were fused if more than 50% of the sequence in the resulting fused region was conserved. We refer to these PhastCons elements located in noncoding regions as conserved non coding sequences (CNSs). Then, we identified conserved RCGTG motifs within these CNSs. A motif was considered conserved when it was present at least in four mammals including human and mouse. Sequences lacking conserved RCGTG motifs were discarded as potential HIF-binding sites (HBS). Finally, sequences containing a conserved motif were scored according to a position specific scoring matrix (PSSM). Aligments and PhasCons elements were downloaded from the UCSC genome browser (Fujita PA et al., 2011) using the February 2009 (hg19) human genome assembly.

11.- Chromatin Immunoprecipitation Assay:

1x10^7 HK-2 cells were prepared for each immunoprecipitation assay. After serum deprivation or hypoxia, crosslinking was performed by adding formaldehyde (1% final concentration).

1x10^7 cells were resuspended in Cell Buffer Mix (10 mM HEPES/KOH, pH 7.9; 85 mM KCl; 1 mM EDTA, pH 8; 1% NP-40) with 1 mM PMSF and Protein inhibitor Mix (Roche Diagnostics). After centrifugation,

pellets were lysed using Nuclear Lysis Buffer (50mM Tris/HCL, pH 7.4; 1% SDS; 0.5% Empigen BB; 10mM EDTA, pH 8).

Glass beads were added (Agilent Technologies, Cat: 200069) and sonication was performed in 15 cycles of 10 sec. with 15% amplitude. After sonication, DNA fragments size was checked by agarose gel analysis. Input (5% of the final volume of the lysates) was separated for further determinations.

Protein concentration of each sample was estimated using the Bradford assay and then adjusted using nuclear lysis buffer. Then, each sample was diluted 1:1.5 with Dilution Buffer (20 mM Tris/HCl, pH 7.4; 100mM NaCl; 2 mM EDTA, pH 8; 0.5%Triton X-100). Preclearing was performed using Sepharose CL-4B beads (Sigma-Aldrich) and incubating for 2 hours at 4ºC. After pre-clearing, 2 µg of each antibody (anti HIF-1α Novus 100-134; anti IgG Millipore 07-690) were added and samples were incubated over night at 4 ºC.

Protein G Agarose beads (Roche Diagnostics) were added to each sample and mixes were incubated for 4 hours at 4ºC. After precipitation and washing, DNA was obtained by treatment with Elution buffer (0.1M $NaHCO_3$; 1% SDS). Crosslink reversal was performed by adding proteinase K (0.5 ug/uL final concentration) and 200 mM NaCl and incubating at 65 ºC over night.

1 µl of purified DNA was used as template for qPCR reaction, using the following primers for the predicted HRE region: Primer pair 1, *forward:* TCG CTG TGA TCA CTG TCT CC*; Reverse:* CTG CCA CAC CCA TAC TCA GA. Primer Pair 2 *Forward*: TCT GCT TCC TTC GGG TTA AA *; Reverse:* CTA GAG AGG CAC GGC ATG AG.

12.- Real Time Monolayer Impedance estimation:

Real Time Cell Analyzer device (Roche Diagnostics) was used for these experiments. Firstly, impedance background was estimated with 100µl of appropriate cell culture medium. Then, 30.000 cells were seeded per well in a final volume of 200µl. Culture impedance was measured every minute during the first 10 hours, then in 5 minutes-intervals during 24 hours and finally every 10 minutes for 72 hours. Each condition was studied in triplicates and monolayer impedance was expressed as Cell Index, calculated by RTCA Software 1.2.

13.- KIF3B 3´UTR cloning and luciferase Assays:

Rattus norvegicus KIF3B (NM_001106529) complete 3´UTR was inserted downstream of the luciferase encoding region of the pGL3-control vector (Promega) using the In-Fusion PCR cloning kit (Clontech-Takara) for mRNA instability assays. pGL3-Control vector was digested with XbaI and KIF3B 3´UTR was amplified from cDNA of NRK-52E cells using specially designed primer pairs which generate 15-bp extremes overlapping with pGL3-control (*forward:* CCG CCC CGA CTC TAG AAG TCC CAA GTA CTG GCA TAG CCT TT ; *Reverse:* GCC GTG TAA TTC TAG CCC AGC ACC CCT CCC AGG). Schemes of the generated constructions as well as used plasmids can be found below.

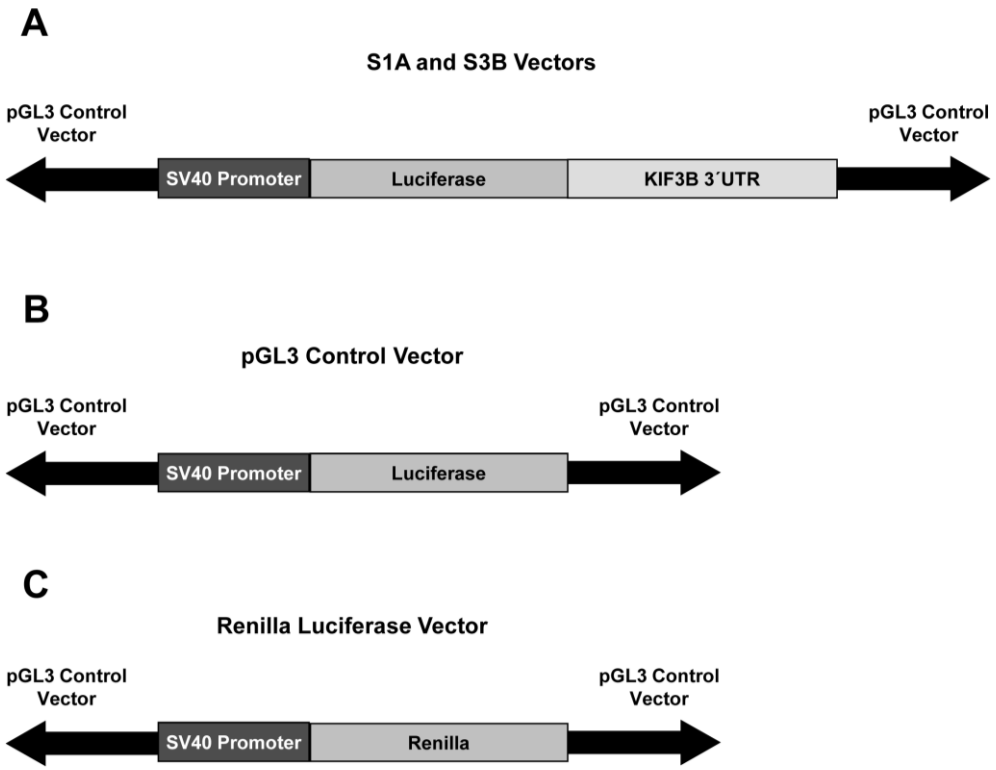

Figure 6: Schematic representation of expression vectors used in reporter assays experiments. (A) Scheme of luciferase-KIF3B 3´UTR vectors S1A and S3B. (B) Empty vector (PGL3 control) used as control in luciferase reporter experiments. (C) Renilla luciferase vector used for normalization.

For luciferase reporter assays, 125,000 NRK-52E cells were seeded in 24 well plates and transfection was performed at 80% confluence. 1 µg of luciferase plasmid and 0.05 ng of renilla luciferase plasmid per well were transfected using 2.5 µl of lipofectamine. Pre-miR-Scramble and Pre-miR-127 were simultaneously transfected with a final concentration of 0.1, 1 and 10 nMolar. Each transfection

condition was carried in triplicate. Luciferase and renilla luciferase activity was measured after 48 hours of transfection using Dual-Luciferase Reporter System (Promega) following manufacturer´s instructions. Luciferase activity was normalized using renilla luciferase values for each sample.

14.- Human serum samples collection and storage:

All experimental desings including human samples have been revised and approved by The Clinical Investigation Ethical Committee of Ramón y Cajal Hospital. After patients or their legal representatives are informed and an informed consent document is signed (See Annex 1) blood samples are collected in 8 ml tubes Vacuette Z sep Clot Activator.

Serum is obtained by centrifugation at 2,500 rpm during 10 minutes. Then it is aliquoted in 250 µl samples and stored at -80 ºC according to the quality standards of the Hospital Ramón y Cajal-IRYCIS Biobank guidelines (ISO 9001). Each sample is anonymous, is identified by an unmistakable code and is associated with a document containing all processing information, such as date and hour of freezing, and relevant clinical data for the study. Samples were obtained after appropriate request following Biobank procedures.

15.- RNA extraction from serum samples:

Total RNA is extracted with miRNeasy mini kit (50) (Qiagen, cat no. 217004) using an optimized protocol for serum samples. 250 µl aliquots are thawed on ice and spin at 1,000g for 5 minutes at 4 ºC to remove debris. 200 µl of precleared samples are used for further steps. For each sample, 800 µl of phenol master mix is prepared, containing 1 µg of MS2 RNA (Roche, cat. No. 10165948001). For data normalization during miRNA detection by qRT-PCR, three synthetic oligonuclotides are also added to this master mix: 25 fmol of synthetic C. elegans miR-39 (sequence: UCACCGGGUGUAAAUCAGCUUG; Sigma-Aldrich), 25 fmol of miR-54 (Sequence: UACCCGUAAUCUUCAUAAUCCGAG; Sigma-Aldrich) (Mitchell PS et al., 2008) and $2x10^6$ copies of Spike-In RNA (Unknown sequence; Exiqon).

750 µl of master mix containing the indicated RNA and housekeepings is added to each 200 µl serum sample and mixed by vortex. The following steps (Phenol/Chloroform extraction and column purification) were carried out as manufacturer´s instructions, except for RPE buffer washing step,

where three repetitions should be done. RNA is eluted from columns adding 50 µl of nuclease free water.

RNA quantity and quality was checked by Agilent Bioanalyzer device using a special kit for small RNA studies (Agilent Technologies). RNA is stored at -80 ºC and only one freeze-thaw cycle is produced prior to miRNA detection by qRT-PCR.

The procedure detailed above is also used for cell culture supernatants. After collection, supernatants are precleared by centrifugation to remove cellular debris and 200 µl are used for total RNA extraction.

16.- microRNA quantification by LNA probes:

Serum circulating miRNAs were detected and quantified by Real-Time PCR using Universal RT miRNA PCR System (Exiqon). 4 µl of the eluted RNA were used as template for universal retrotranscription reaction with a final volume of 20 µl. Mixtures were incubated at 42 ºC for 60 minutes and 95 ºC for 5 minutes and immediately cooled down to 4 ºC. cDNA was diluted 1/11 with nuclease-free sterile water and 4 µl were used as template for PCR reaction.

Real time PCR detection was performed using SYBR Green and specific probes, commercially available (Exiqon), for each miRNA of interest. Master Mix preparation and temperature protocol were performed following manufacturer´s instructions and temperature protocol was performed as follows: enzyme activation at 95ºC for 10 min.; 45 cycles of 95ºC for 15 sec. and 60ºC for 60 sec. All reactions were carried out in triplicates in a Light Cycler 480 equipment (Roche) and Cq values were calculated with 2nd derivative method (Light Cycler 480 Software 1.5, Roche). miRNA expression values are expressed as ΔCq, obtained from the following formula:

ΔCq= miRNA Cq - Spike In Cq

17.- microRNA qRT-PCR Array and data analysis:

Circulating miRNA profiling experiments were carried out using Taqman Low Density Arrays for miRNAs (Applied Biosystems). For each sample, total RNA from three different aliquots was extracted as detailed above to avoid false results due to aliquot variability or RNA extraction procedure. After RNA quantification and quality checking, RNA samples were pooled for further steps.

Retrotranscription was performed using Megaplex RT primer pool panel A and B and Taqman miRNA retrotranscription kit components (Applied Biosystems) following manufacturer´s indications. Panels A and B were used to cover a wider range of miRNAs, which includes a total of 754 assays. 3µl of RNA were used as template for this reaction.

PCR determinations were performed using Taqman Array Human microRNA cards (Applied Biosystems). Two cards were run for each sample, corresponding to panel A and B. For each reaction, 6 µl of retrotranscription product were used as template and PCR reactions were performed following manufacturer's instructions.

All reactions were carried out in a 7900HT Fast Real-Time PCR System device (Applied Biosystems) and raw Cq were obtained using SDS v2.3 software (Applied Biosystems). Baseline and threshold settings were carefully optimized and maintained during the whole experiment to assure comparable results among the different samples and arrays.

Raw Cq values were exported and analyzed in Excel (Microsoft). Schematic representation of analysis workflow can be found in Figure 7. Firstly, miRNAs not expressed in any sample were eliminated. After this step, all values showed as "undetermined" were substituted by 40 as Cq value. Next, ΔCq values were calculated (ΔCq= miRNA Cq - Housekeeping Cq)using as housekeeping control the mean of the Cq values of all expressed miRNAs (Cq values <35) (Mestdagh P. et al., 2009). Next, miRNA with inconsistent ΔCq values between control samples (ΔΔCq>1) were also excluded from the study.

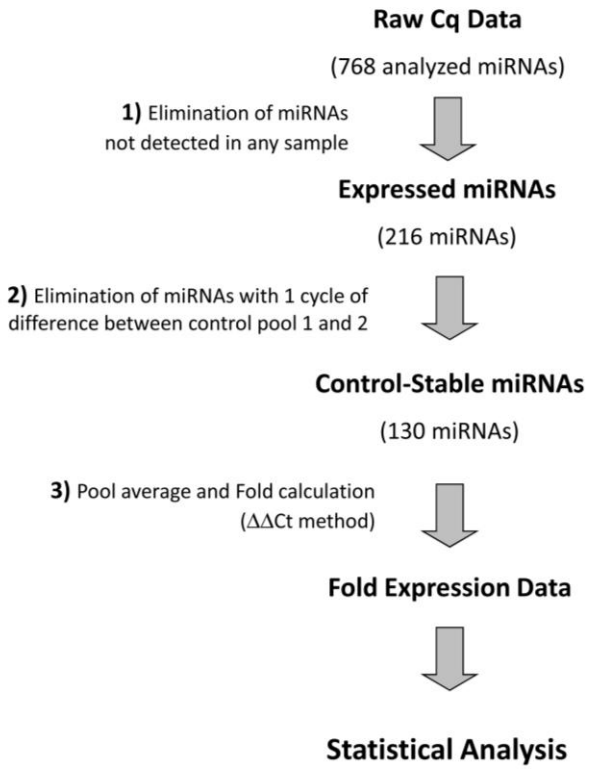

Figure 7: qRT-PCR array analysis workflow: Schematic representation of raw data management and miRNA selection steps.

After these initial selection steps, based on technical criteria, fold changes were calculated using $2^{-\Delta\Delta Cq}$ method. Downregulated miRNAs values (Folds<1) were transformed into negative values applying the following equation: transformed fold value= -1/fold value. After application of statistical tests, only miRNAs showing statistically significant changes over ±2 folds were taken into account for further steps.

For final selection, miRNAs showing most prominent and significant changes were submitted to functional analysis based on their predicted target genes. For each miRNA, potential targets were downloaded from Targetscan Human 5.1 database (http://www.targetscan.org/vert_50/; Friedman RC. et al., 2009). Target gene list were then analyzed using the online Bioinformatic Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/tools.jsp; Huang da W. et al., 2009). Functional annotation tool was used with human genome background and default settings. Target genes were classified into functional categories of Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Statistical analysis was performed to indicate whose functional categories are enriched in the target gene list compared to their relative abundance in the whole human genome. Only miRNA whose target genes were enriched in functional categories relevant for our study context were taken into account for selection of the final panel of miRNAs.

18.- Patient Cohorts:

18.1. Serum circulating miRNA profiling experiments:

For this experiment, 10 healthy subjects and 4 AKI patients were included, divided into the following groups:

- Two pools (n=5 individual each) of healthy controls: Volunteers without pathologies or pharmacological treatment. Distribution of individuals between both pools was performed matching age mean, proportion of men/women and proportion of smokers/non smokers.
- AKI patients with different etiologies were selected from AKI patient collection reference **AD4-FRA**, stored in Ramon y Cajal Hospital-IRYCIS Biobank, including:
    - 2 patients with Ischemic AKI. For each one, two samples were used: one at the day of diagnosis (Day 0) and other when renal function is recovered (Days 7-10).

- 1 patient withToxic AKI. Two samples were used: one at the day of diagnosis (Day 0) and other when renal function is recovered (2 Months).
- 1 Patient with Septic AKI. Only sample at the day of diagnosis was included.

18.2. ICU AKI Patients:

For this experiment healthy controls and ICU patients with AKI were obtained from collections **AD-5-BCS** and **AD1-FMO** respectively, stored in Ramon y Cajal Hospital-IRYCIS Biobank. 55 individual were analyzed, divided in the following groups:

- 20 Healthy controls without pathologies.
- 35 ICU patients with AKI, including:
  - 19 patients presenting intrinsic AKI (ATN).
  - 16 patients with pre-renal AKI.

In this cohort, AKI was diagnosed using AKIN criteria. For this purpose, only changes in serum creatinine were taken into account. Patients were considered having pre-renal azotemia when renal function returns to baseline serum creatinine after adequate treatment in least than 72 hours. For each patient, we have analyzed serum samples collected at day 0 (Diagnosis), day 1, day 2, day 3 and day 7 of evolution, when available.

18.3. Cardiac Surgery with Cardiopulmonary Bypass Patients:

For these experiments, we have collected a cohort of adult and pediatric patients who underwent cardiac surgery with CPB. This patient sample collection is stored in Ramon y Cajal Hospital-IRYCIS Biobank with reference **AD3-CEC**.We have analyzed 39 patients organized in the following groups:

- **IA:** 10 adult patients with 0-2 points in Thakar system.
- **IB:** 10 pediatric naïve patients operated with CPB.
- **II:** 15 adult patients with altered baseline renal function and > 5 points in Thakar system.
- **III:** 4 adult patients with normal renal function and > 5 points in Thakar system.

Thakar system graded the preoperative risk of AKI development after cardiac surgery (Thakar CV et al., 2005). In this set of patients, AKI was diagnosed using RIFLE, AKIN and creatinine kinetics criteria independently. As in previous cohorts, only changes in sCr were considered. For each patient, we have analyzed serum samples at different time-points: Basal (before surgery), Immediate post-surgery (IPS), 24, 48, 72 hours and 7 days after surgery.

19.- Statistical Analysis:

Data are presented as mean±s.e.m in case of *in vitro* and *in vivo* experimental model data. Median and first and third quartiles are presented for patient miRNA expression data.

For experimental models data and patient results, statistical analyses were performed as follows: Shapiro-Wilk test was first performed to confirm or reject normal distribution. After the Levene test for homogeneity of variance, T-Test and ANOVA with post hoc Bonferroni correction were used for normal samples. For non-normal distributed data, the following non parametric analysis were used: Kruskal–Wallis test for group comparison and for intergroup differences post hoc Mann–Whitney U-tests. $P<0.05$ was considered significant. Spearman Rho correlation coefficient was used for correlation studies between numerical variables.

Hierarchical clustering analysis using squared Euclidean distance method was employed for patient group classification in massive screening analysis. Receiver-Operator Characteristic (ROC) curve test was performed to evaluate sensitivity and specificity of miRNAs as AKI biomarkers. Statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software version 19.0.

RESULTS

1. - miR-127 is induced in response to H/R and I/R:

As miRNAs are dynamic regulators of cell responses to environmental stimuli and stress (Krol J et al., 2010), we hypothesized that the regulation of the expression of these molecules could be involved in the proximal tubule response to I/R injury. For this purpose, previous work of our lab performed a massive screening experiment, using hybridization microarrays, in rat proximal tubule cells (NRK-52E) submitted to H/R. As it is detailed in material and methods section, H/R protocol closely mimics the stimuli to which proximal tubule cells are exposed during I/R *in vivo*. This massive screening experiment led to a set of miRNAs that modulated their expression during hypoxia and reoxygenation (Table 2).

TABLE 2: Differentially expressed miRNAs in NRK-52E cells submitted to H/R.

Control vs Hypoxia		Hypoxia vs Reoxygenation	
miRNA	**Fold change**	**miRNA**	**Fold change**
rno-miR-101a	**1**	**rno-miR-101a**	**2,63**
rno-miR-127	**1,16**	**rno-miR-127**	**2,46**
rno-miR-129*	**1**	**rno-miR-129***	**2,13**
rno-miR-154	**1**	**rno-miR-154**	**8,03**
rno-miR-28	**1,27**	**rno-miR-28**	**1,90**
rno-miR-376b	**1,33**	**rno-miR-376b**	**6,70**
rno-miR-223	**1**	**rno-miR-223**	**-1,91**

In this work, we validated these initial microarray data by real time PCR (qRT-PCR) using taqman assays. Data are expressed as fold change, calculated by $2^{-\Delta\Delta Ct}$ method comparing each sample to Normoxia (Nx). From the set of miRNAs which significantly modulate their expression under H/R, rno-miR-127 was the most consistent and significantly modulated miRNA in NRK-52E cells, showing an increased expression during minimum medium hypoxia (mimicking ischemia) and at 1 hour of reperfusion (Figure 8A).

In the next step, we wanted to assess if miR-127 modulation was conserved among species. Thus we studied the expression of the human homolog of rno-miR-127 (hsa-miR-127-3p) in the human proximal tubule cell line HK-2 submitted to H/R protocol. qRT-PCR data demonstrated that hsa-miR-

127-3p is also induced in human cells, but showing a different expression pattern. In this case, we found increased expression during complete medium hypoxia and along reoxygenation (Figure 8A, lower panel). Differential regulation of miR-127 between species could be underlying the different expression pattern between human and rat, as we will discuss later.

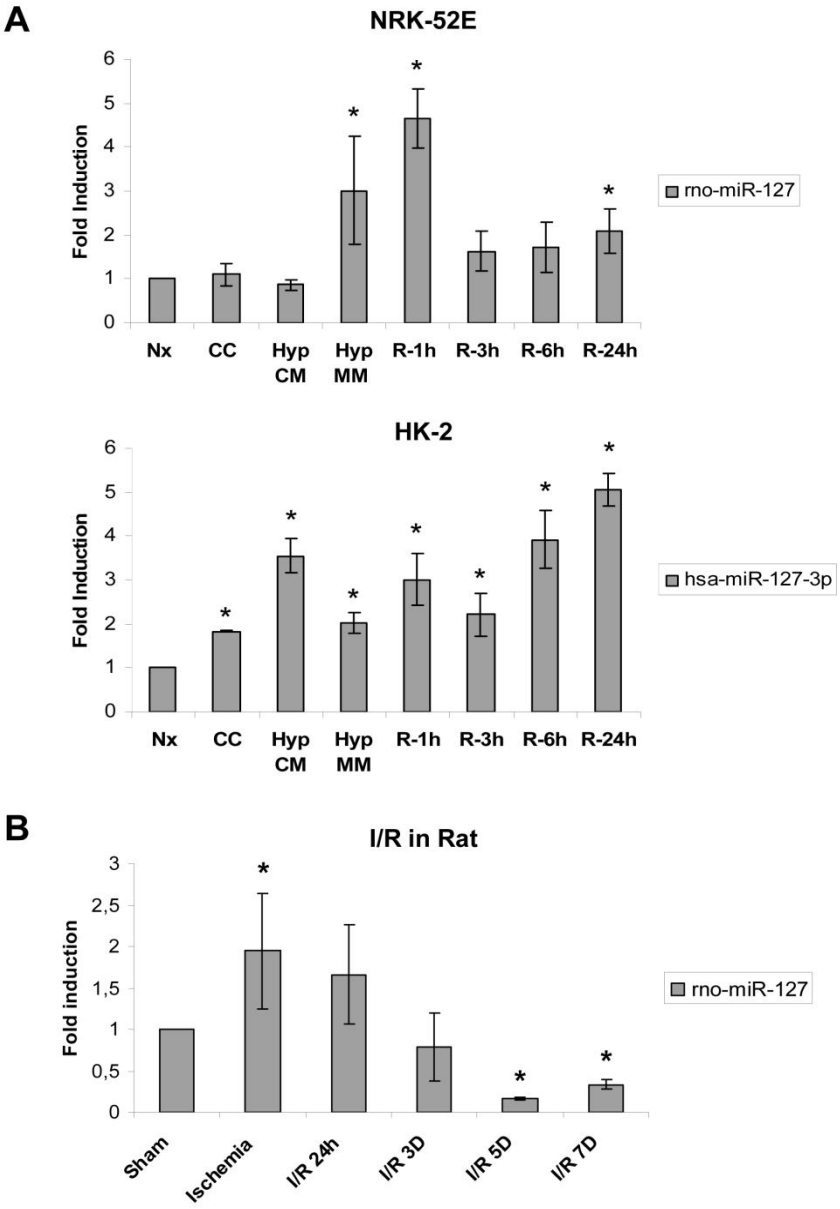

Figure 8: miR-127 is modulated in response to Hypoxia/Reoxygenation *in vitro* and during Ischemia/Reperfusion *in vivo.* (A) rno-miR-127 modulation in rat proximal tubule cells NRK-52E (Upper panel) and human proximal tubule cells HK-2 (Lower panel) submitted to H/R protocol. Data are presented as mean ± s.e.m. of five independent experiments. Asterisks indicate statistical significance comparing each sample with Normoxia (Nx) ($P<0.05$). (B) rno-miR-127 modulation in rat kidney in response to ischemia/reperfusion (I/R). Data are presented as mean ± s.e.m. of at least five animals per condition. Asterisks indicate statistical significance ($P<0.05$) comparing each experimental group to Sham condition.

In addition, we studied whether this induction could be also observed in our *in vivo* model of renal I/R in rat. In this model, proximal tubule cell damage is observed during reperfusion followed by renal dysfunction. As can be observed in figure 8B, rno-miR-127 is induced after ischemia and at 24 hours of reperfusion, time-point when tissue and renal function damage is maximal (Conde E et al, 2012).

Taken together, these data indicate that miR-127 is modulated in proximal tubule cells and renal tissue in response to H/R and I/R, respectively.

2. - hsa-miR-127 is regulated during H/R by HIF-1α:

Once we have observed that miR-127 is modulated during H/R and I/R, we went further into miR-127 regulation. As indicated before, HIF is the key regulator of transcriptional responses to hypoxia (Rocha S., 2007) and HIF-1α is the main isoform expressed in kidney proximal tubule cells. Based on this evidence, we hypothesized that this subunit could be a regulator of miR-127 induction in our *in vitro* system.

To assess this issue, we performed HIF-1α knockdown experiments by siRNA transfection in HK-2 cells, since HIF-1α knockdown did not work in NRK-52E cells. Previous work of our lab demonstrated that in our *in vitro* model, HIF-1α is expressed not only during hypoxia, but also at several time points during reperfusion, showing a biphasic induction pattern (Figure 9A) (Conde E. et al 2012).

We studied hsa-miR-127 expression by qRT-PCR in HK-2 cells transfected with specific HIF-1α siRNA and scramble control. Data are presented as fold change values, using scramble normoxia as reference. microRNA expression values are compared between scramble and siRNA for each condition. As shown in Figure 9B, HIF-1α interference successfully prevented hsa-miR-127 induction in complete medium hypoxia (Hyp CM) and 1 hour of reperfusion. HIF-1α knockdown control western blot demonstrating significant HIF-1α inhibition is shown in Figure 9C.

These interference results demonstrate that HIF-1α is a regulator of miR-127-3p expression in HK-2 cells during H/R.

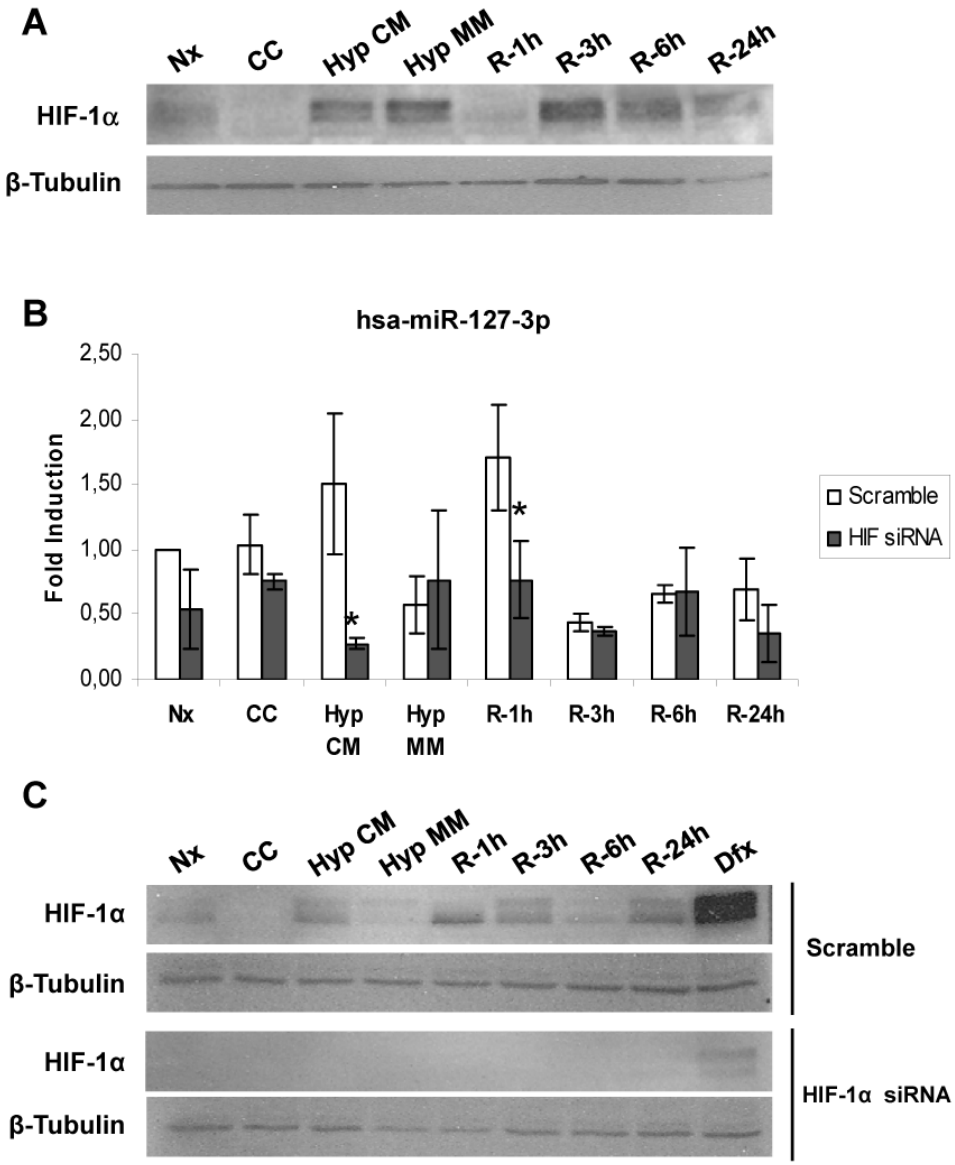

Figure 9: HIF-1α regulates miR-127-3p in HK-2 cells in response to Hypoxia/Reoxygenation. (A) HIF-1α stabilization in HK-2 cells during H/R protocol was estimated by western blot using β-tubulin as loading control (lower panel). Representative image from five independent experiments is shown. (B) hsa-miR-127-3p expression in HK-2 cells transfected with scramble (White bars) or HIF-1α siRNA (Black bars). microRNA expression was determined by quantitative PCR and fold values were obtained comparing each sample to Normoxia scramble (Nx). Data are presented as mean ± s.e.m. of five independent experiments. Asterisks indicate statistical significance ($P<0.05$) comparing scramble to siRNA values in each condition. (C) Interference efficiency was estimated by HIF-1α detection by western blot. Upper panel shows HIF-1α stabilization in scramble transfected cells submitted to H/R whereas lower panel indicates HIF-1α protein levels in siRNA transfected cells. β-tubulin was used as loading control. Deferoxamine was used as a positive control for HIF-1α stabilization. Representative western blot image from five experiments is shown.

Trying to characterize miR-127-3p regulation by HIF-1α, in the next step, we studied if this regulation tood place by direct binding of this transcription factor to putative HRE elements located close to miR-127-3p sequence.

First, we performed, by bioinformatics approaches, a HRE prediction study in the genomic region where hsa-miR-127-3p gene is located. This prediction, based on sequence alignment, conservation and a position scoring matrix data, identified a potential functional HRE downstream miR-127-3p sequence (Figure 10). This element was conserved among vertebrates and was located in a CpG island, making it a good candidate for miR-127 regulation.

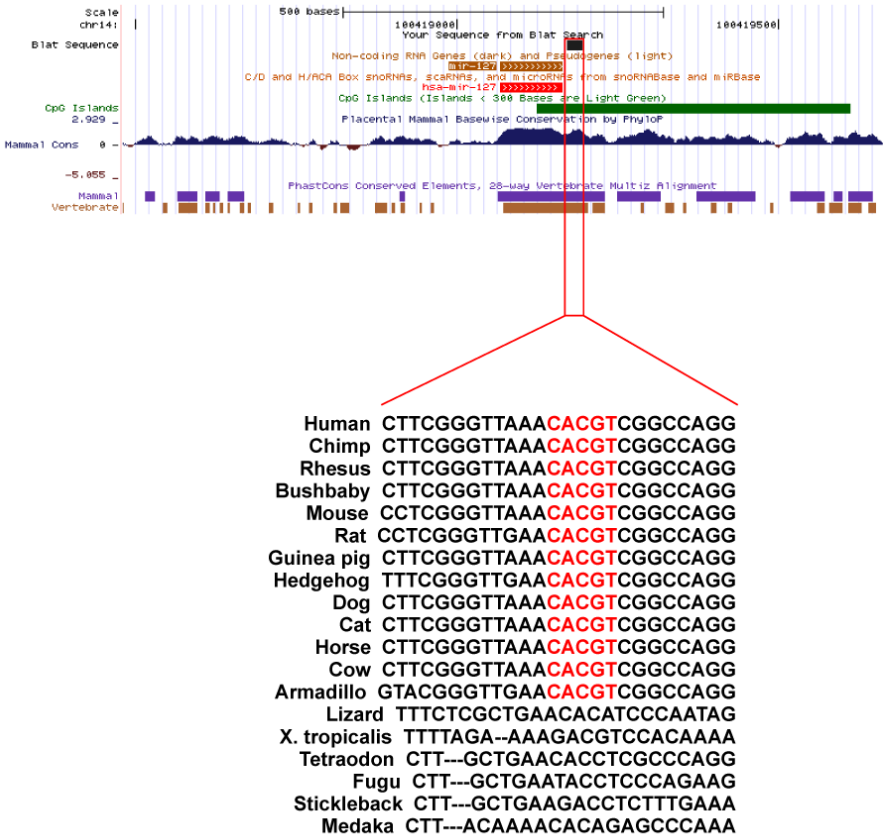

Figure 10: miR-127 gene DNA region presents a putative Hypoxia Response Element (HRE). Bioinformatics sequence alignment and conservation studies detected a consensus HRE sequence (CACGT) downstream miR-127 coding region. Alignment map (upper part) shows miR-127 gene and HRE element location into human genomic DNA. Sequence scheme (lower part) indicates that this HRE element (marked in red) is conserved among mammals and several vertebrate species.

To demonstrate whether HIF-1α directly binds to this predicted HRE element, we performed Chromatin Immunoprecipitation (ChIP) assays in HK-2 cells submitted to H/R. In this method, protein and chromatin crosslinking is performed and HIF-1α is precipitated using a specific antibody. The precipitation of the transcription factor draws the chromatin to which the factor is bound to manage its transcriptional regulation. After precipitation, crosslinking is reversed and the chromatin is isolated. Then, the region of interest can be studied by qRT-PCR using specific primers. Results are represented as percentage of input (samples where any precipitation or antibody treatment has been done) and IgG antibody is used as technical negative control.

Two primer pairs were designed to study the predicted HRE element (Figure 11A), but as can be observed in figure 11B, after HIF-1α antibody was used for precipitation, none of the studied sequences increased their relative concentration during hypoxia, compared to normoxia condition. These data indicated that HIF-1α does not directly bind to the predicted HRE element.

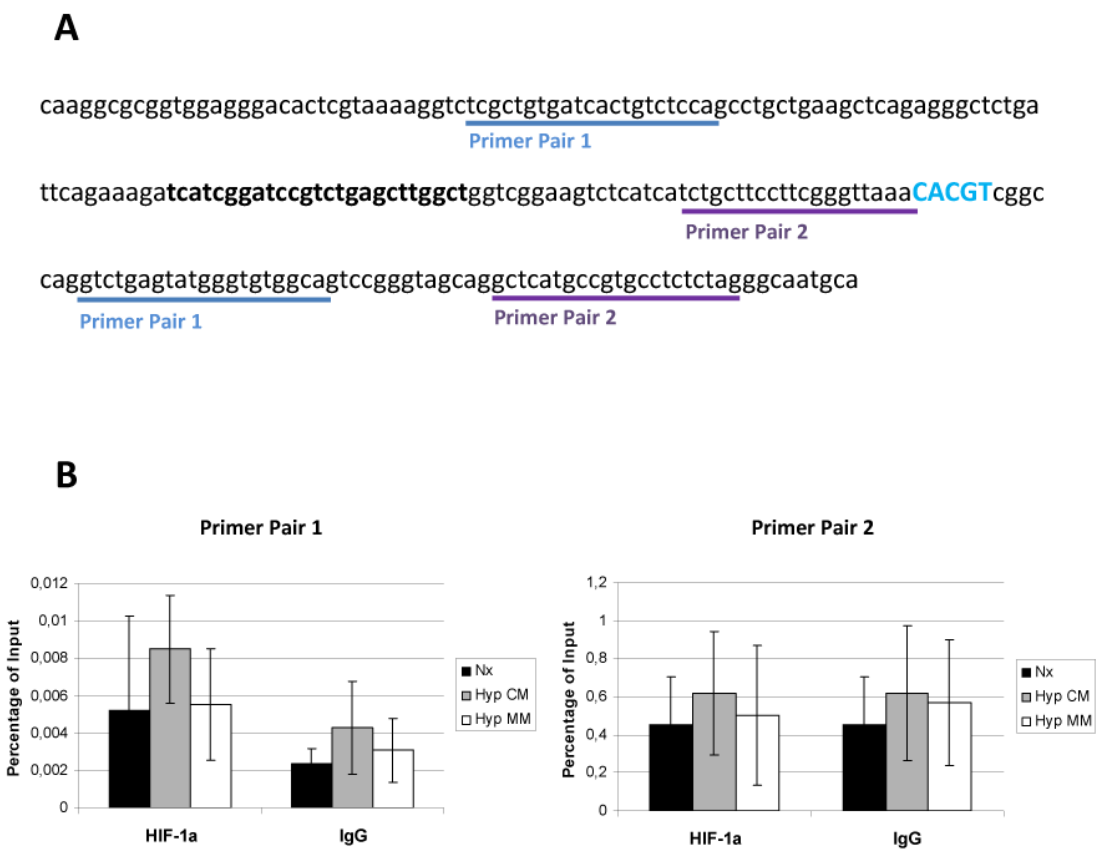

Figure 11: HIF-1α does not directly bind to the predicted HRE element. Chromatin immunoprecipitation assays were performed and target sequence containing the predicted HRE element was studied by qRT-PCR using two different primer pairs (A) Primer pair aligment with genomic DNA. miR-127-3p mature sequence is marked in bold and predicted HRE element is highlighted in blue. (B) qRT-PCR data are presented as mean±SEM of percentage of input of two independent experiments. IgG antibody was used as negative control for precipitation.

Taken together, these data demonstrate that HIF-1α is a regulator of hsa-miR-127 in our *in vitro* system. However, the functional HRE for HIF-1α binding could not be successfully identified in our study. This could indicate that the active HRE elements involved in miR-127-3p regulation could be located far from the miRNA sequence or that HIF-1α transcription regulation could be indirect, as it will be further discussed.

3. - rno-miR-127 modulation leads to changes in cell adhesion and cytoskeleton structure:

After studying the modulation and regulation of miR-127 in our system, we wanted to assess the biological significance of induction of this miRNA during H/R. Previous work of our lab demonstrated that H/R injury leads to proximal tubule cell adhesion alterations by cytoskeleton disorganization and redistribution and disassembly of adhesion complexes (Sáenz-Morales D et al., 2006). Based on these data, we hypothesized whether miR-127 modulation could regulate cell adhesion during H/R.

Firstly, we performed adhesion assays in NRK-52E cells where miR-127 expression is modulated by transfection of Pre-miR-127, Anti-miR-127 and their corresponding scramble controls (Pre-miR-sc and Anti-miR-sc). Cell adhesion was estimated by monolayer impedance, measured by Real Time Cell Analyzer (RTCA) device.

RTCA plates contain 16 cell culture wells whose bottom is covered by interdigitated micro-electrodes. The presence of cells on top of the electrodes alters the local ionic environment at the electrode/solution interface, leading to an increase in the electrode impedance. Thus, the more cells are attached on the wells, the higher electrode impedance. In addition, impedance depends on the cell number as well as the quality of the cell interaction with the electrodes. Thus, increased cell adhesion or spreading will lead to a larger change in electrode impedance. Therefore, electrode impedance can be used to monitor cell number, morphology, and adhesion degree.

Impedance is expressed as arbitrary Cell Index (CI) units, calculated by RTCA software. The CI at each time point is defined as (Rn-Rb)/15; where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the media alone.

Adhesion assays were performed under normoxic and H/R conditions. For normoxic measurement, cells were seeded in RTCA plates 24 hours after transfection and real time measurement of cell culture impedance was performed. For hypoxia condition transfected NRK-52E cells were submitted to serum starvation and hypoxia. Immediately after hypoxic treatment, they were detached and

seeded in RTCA plates to allow impedance measurement during reperfusion. Each transfection condition was measured in triplicate.

As can be observed in Figure 12, rno-miR-127 overexpression by pre-miR transfection increases monolayer impedance under normoxic conditions and during reperfusion.

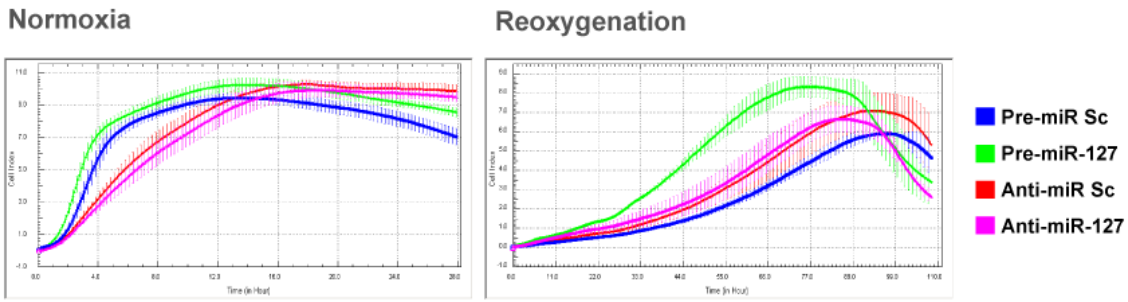

Figure 12: miR-127 overexpression promotes cell adhesion during Normoxia and Reoxygenation. NRK-52E cells were transfected with pre-miR-127, anti-miR-127 and their respective scramble control. Adhesion was estimated by measuring monolayer impedance with RTCA device under Nomoxic and Reoxygention conditions. Each transfection condition was measured in triplicate and cell index is presented as mean±s.e.m. Representative images from 3 independent experiments are shown.

Based on these initial data, we wanted to confirm whether an increase in cell impedance was associated to cell adhesion promotion by miR-127. To asses this issue, we studied, by immunofluorescence staining, proximal tubule epithelial cell-matrix and cell-cell adhesion structures.

Ischemic injury leads to actin cytoskeleton remodeling and stress fibers formation. Actin cytoskeleton disorganization contributes to loss of cell polarity and disassembly of adhesion structures such as Focal Adhesion complexes (FAC) (Sáenz-Morales D et al., 2006). As indicated in previous sections of this work, these complexes are composed by transmembrane proteins called Integrins, α-actin and intermediary proteins such as Paxillin and are responsible for cell adhesion to extracellular matrix.

We performed fluorescence staining for actin (red) and immunofluorescence for Paxillin (green) in NRK-52E cells transfected with pre/Anti-miR-127 and submitted to H/R. As can be observed in Figure 13, miR-127 overexpression prevents actin cytoskeleton disorganization and promotes actin-paxillin co-localization (Yellow colour in marked circles), indicating FAC correct assembly. Moreover, miR-127

blockade by anti-miR aggravates cytoskeleton and adhesion structures disorganization caused by hypoxia.

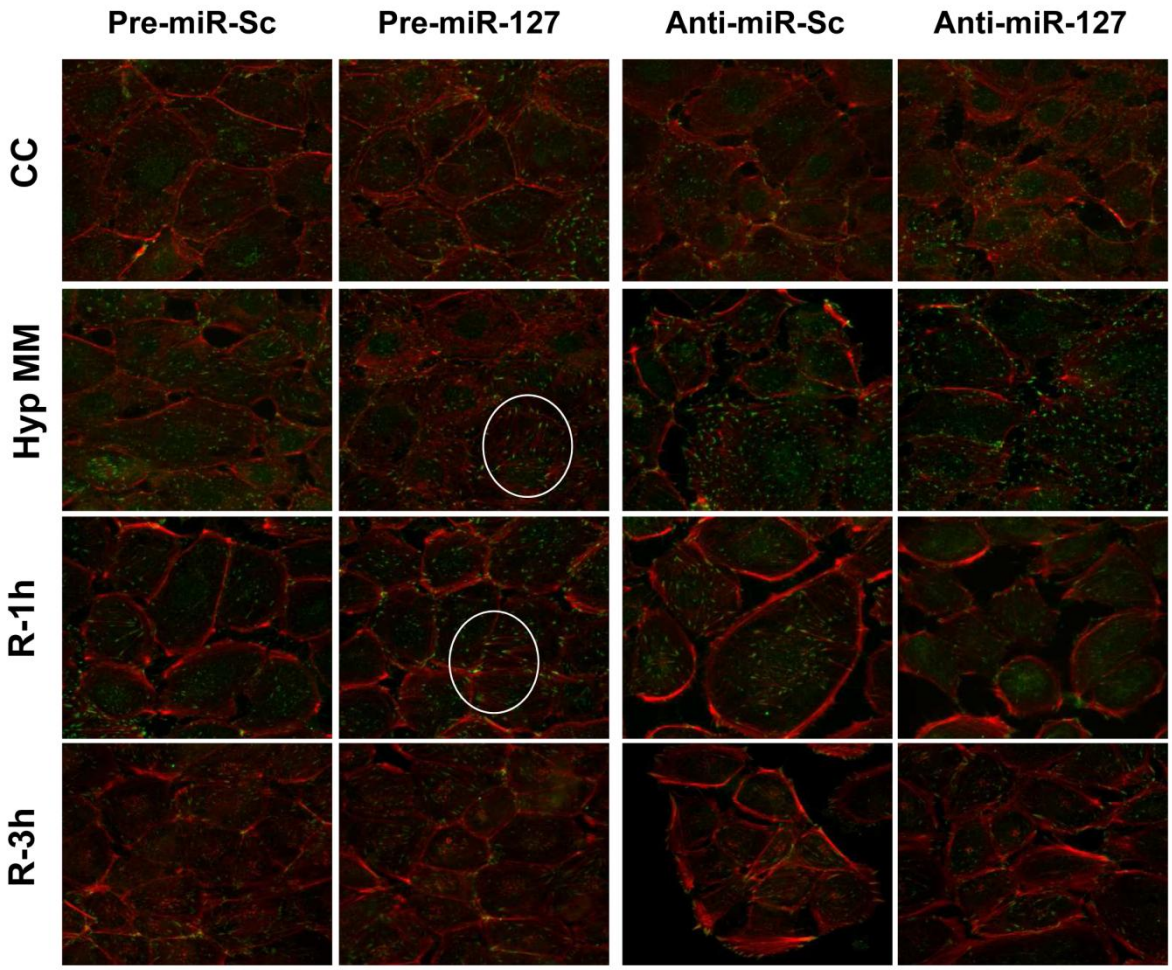

Figure 13: miR-127 overexpression protects actin cytoskeleton organization and focal adhesion complexes assembly during H/R: Fluorescence staining and immunofluorescence to detect actin cytoskeleton organization (Red) and paxillin localization (Green) was performed in transfected NRK-52E cells. Representative confocal microscopy images from three experiments are shown. Co-localization paxilin/actin, as indication of FAC assembly, is marked by circles. Magnification 400X.

On the other hand, tight junctions (TJ) are essential for epithelial barrier impermeability and they are disrupted by H/R injury, as we previously demonstrated (Sáenz-Morales D et al., 2006).TJ are composed by a network of sealing strands formed by transmembrane proteins, mainly claudins and occludins, embedded in both plasma membranes. These proteins associate with different peripheral membrane proteins responsible for anchoring TJ strands to the actin cytoskeleton, including the zonula occludens-1 protein (ZO-1).

We investigated the effects of rno-miR-127 modulation in TJ integrity by immunofluorescence of ZO-1. In normal conditions, ZO-1 presents a continuous staining along the plasma membrane of proximal tubule cells. However, this continuous distribution is lost during H/R. Anti-miR transfection clearly enhances hypoxic damage increasing ZO-1 redistribution from the membrane to the cytoplasm, resulting in a discontinuous signal along the membrane and leading to the appearance of gaps among epithelial cells (Figure 14).

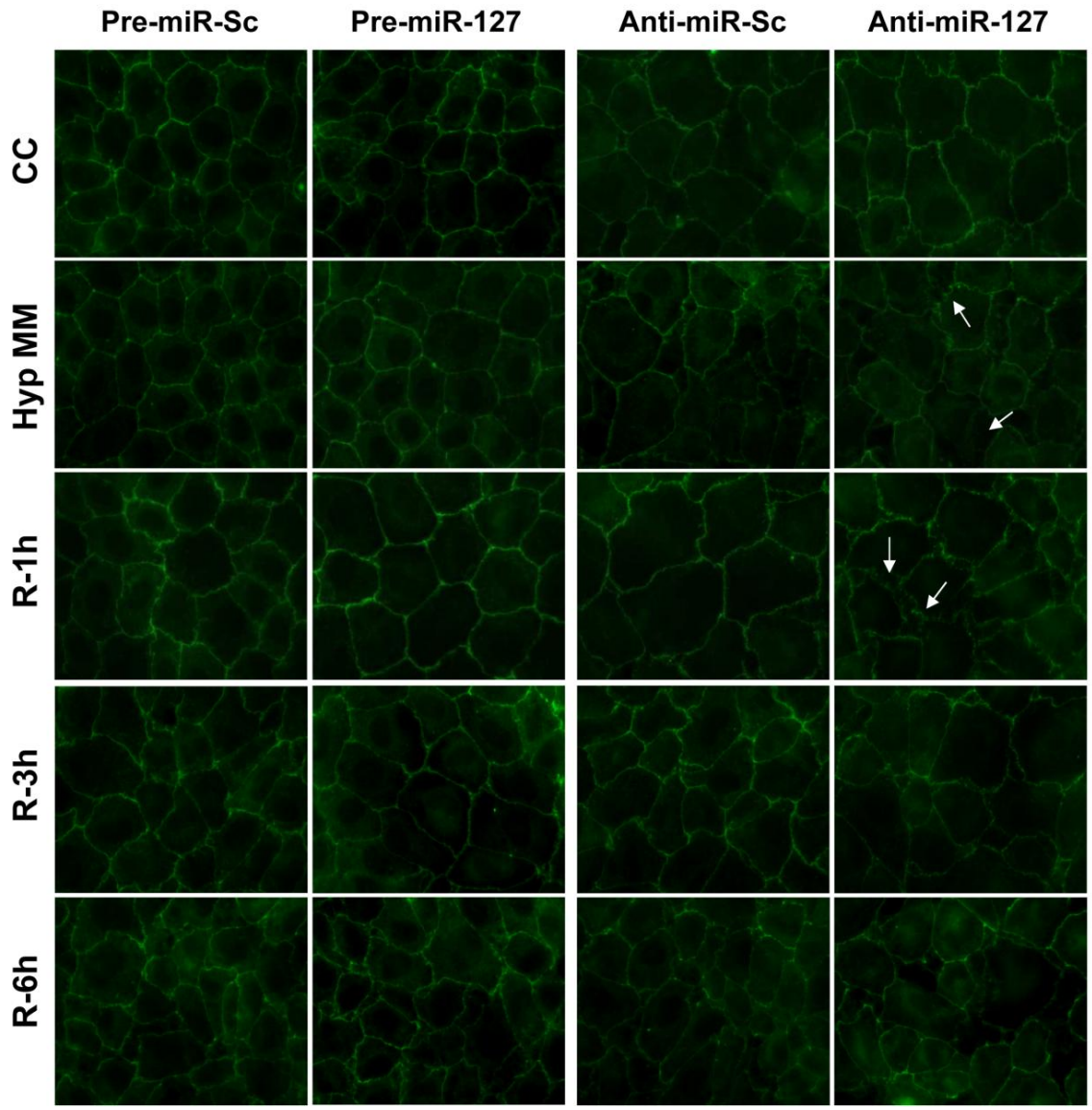

Figure 14: miR-127 overexpression *in vitro* abrogates tight junction disruption during H/R. NRK-52E cells were transfected with pre-miR-127, anti-miR-127 and their respective scramble controls. 24 hours after transfection, they underwent H/R protocol. ZO-1 immunofluorescence was used to study tight junction integrity. Representative confocal microscopy images are presented from three independent experiments. ZO-1 redistribution from plasma membrane as indication of TJ disruption is marked by arrows. Magnification 400X.

In summary, all these data demonstrate that rno-miR-127 induction promotes maintenance of proximal tubule structure and function during H/R by preventing cell adhesion structures alterations and maintaining cell monolayer integrity.

4. - Kinesin Family Member 3B (KIF3B) is a rno-miR-127 target in rat proximal tubule cells during H/R:

To go further into the biological significance of rno-miR-127 induction, we performed a bioinformatics target prediction study using the databases available online: microcosm (Griffiths-Jones S. 2008) (www.ebi.ac.uk/enright-srv/**microcosm**), Targetscan 4.1 (Friedman RC. et al., 2009) (www.**targetscan**.org/vert_40/) and Pictar I (Krek A. et al., 2005) (www.pictar.mdc-berlin.de).

These target prediction programs base their algorithms in sequence complementarity between the 5´seed region of the miRNA (bases 2 to 8) and the 3´UTR of the target gene. To improve the likelihood of target recognition other parameters are taken into account for prediction, such as the target site conservation among species, the thermodynamic stability of the mRNA/miRNA duplex and the absence of secondary structures surrounding the miRNA binding site.

However, in addition to these common characteristics, there are some differences among the several approaches. Both TargetScan and Pictar I take into account evolutionary conservation of target sites for their predictions. However, Pictar I allows both perfect and imperfect seed complementarity, always avoiding G-U pairing. TargetScan includes a “context score” including features such as local A-U content and target site position in the UTR. microCosm is the less restrictive algorithm because it does not require exact seed complementarity and their predictions include G-U pairing (van Rooij E. 2011).

Due to these differences in prediction algorithms, results from the three databases were compared and only those genes included in at least two databases were taken into account. Combining results from several databases to look for overlapping predictions has been demonstrated as the strategy with the highest sensitivity when compared with experimentally validated data sets (van Rooij E. 2011). We finally chose KIF3B and MAPK-4 for further studies due to their potential biological significance in our system since KIF3B is involved in cellular trafficking (Reed AA. et al., 2010), and MAPK-4 has been involved in microtubule cytoskeleton organization (Beck M et al., 2010). However, MAPK-4 did not finally behave as a real target of miR-127 in our system (data not shown) and we focus our studies in KIF3B.

Firstly, we wanted to assess if this protein was modulated in proximal tubule cells during H/R. For this purpose, we studied mRNA and protein levels of KIF3B by qRT-PCR and western blot respectively in NRK-52E cells under H/R. KIF3B mRNA levels were reduced during minimum medium hypoxia and 1 hour of reperfusion in NRK-52E cells, time-points when miR-127 is induced. Similar expression pattern could be observed at protein level (Figure 15).

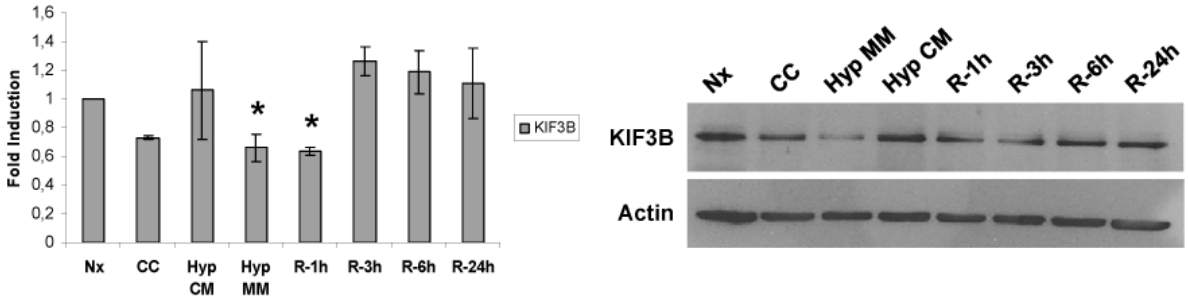

Figure 15: KIF3B is modulated in NRK-52E cells submitted to H/R. KIF3B mRNA and protein levels were estimated in NRK-52E cells submitted to H/R. mRNA levels were determined by quantitative PCR. Fold changes were calculated using Normoxia (Nx) as basal condition. Data are presented as mean ± s.e.m. of five independent experiments and asterisks indicate statistical significance ($P<0.05$) when each sample is compared to normoxia. KIF3B protein levels were estimated by western blot using actin as loading control. Representative blot from five different experiments is shown.

Moreover, we performed Pre/Anti-miR transfection experiments to determine whether modulation of miR-127 could regulate KIF3B expression. Although significant changes were not found for mRNA studies, since miRNAs in animal cells could work through blocking mRNA translation, miR-127 overexpression and inhibition modulate KIF3B protein (Figure 16). KIF3B levels are decreased when miR-127 is overexpressed, particularly during Control Condition (CC) and minimum medium hypoxia (Hyp MM). These results identify for the first time KIF3B as a real target of miR-127 in our system.

To further confirm KIF3B modulation by miR-127, KIF3B 3´UTR was cloned into luciferase vectors and mRNA destabilization assays were carried out. For this experimental approach, luciferase gene expression is driven by SV-40 promoter and KIF3B 3´UTR is cloned after luciferase gene (Figure 6, Material and Methods section). This vector configuration produces high expression levels of luciferase in basal conditions. However, after miRNA overexpression by pre-miR transfection, luciferase expression levels will be reduced by mRNA degradation or translation inhibition if the miRNA binds to the cloned UTR.

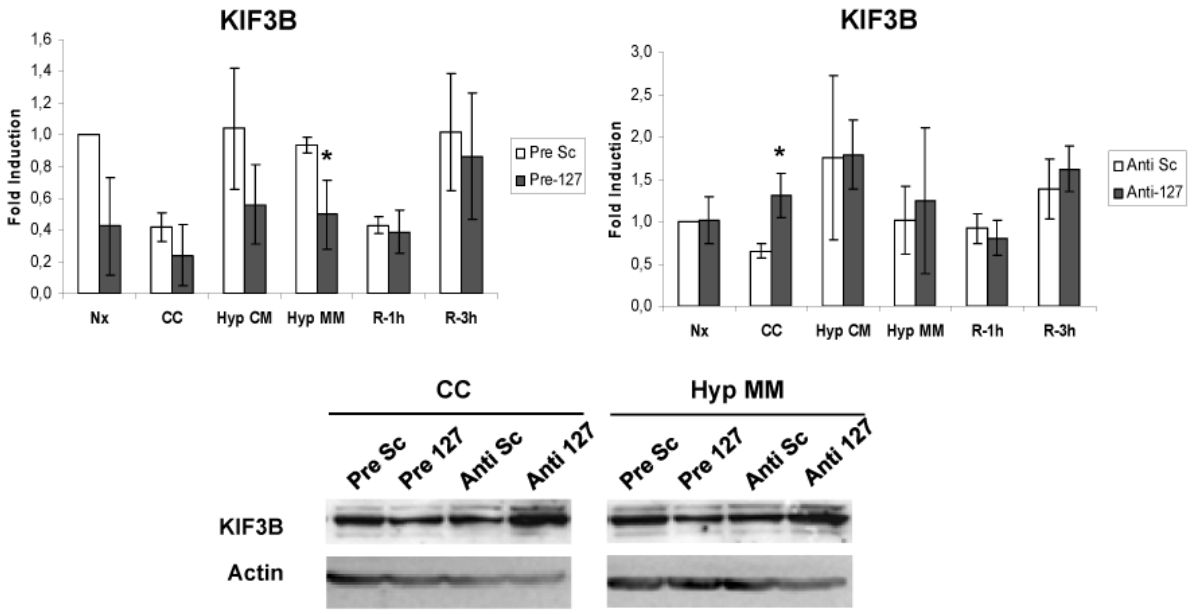

Figure 16: KIF3B is modulated in response to miR-127 overexpression and inhibition. Study of KIF3B mRNA and protein levels in NRK-52E cells transfected with pre/Anti-miR-127. KIF3B mRNA was estimated by qRT-PCR in cells treated with pre-miR-127 (left panel) and anti-miR-127 (Right panel). Fold change were calculated using Nx Scramble as reference condition. KIF3B protein levels were assessed by western blotting using actin as loading control. Illustrative blots from five independent experiments are shown.

Two independent constructions, named as S1A and S3B, were generated to avoid possible side-effects due to mutations undetected by sequencing or other unpredictable effects of cloning procedure (Figure 6, Material and Methods section). Both UTR constructions and pGL3 Control empty vector were transfected with different concentrations of Pre-miR-127 in NRK-52E cells. Renilla luciferase activity was used for normalization control.

Data are shown as percentage of luciferase activity reduction by Pre-miR-127 in comparison to scramble control (Scramble=100). miR-127 overexpression significantly reduces luciferase activity in both UTR vectors in a dose-dependent manner, demonstrating that this miRNA directly regulates KIF3B expression through recognition of its 3´UTR. Therefore, KIF3B is a real target of miR-127 in our system (Figure 17).

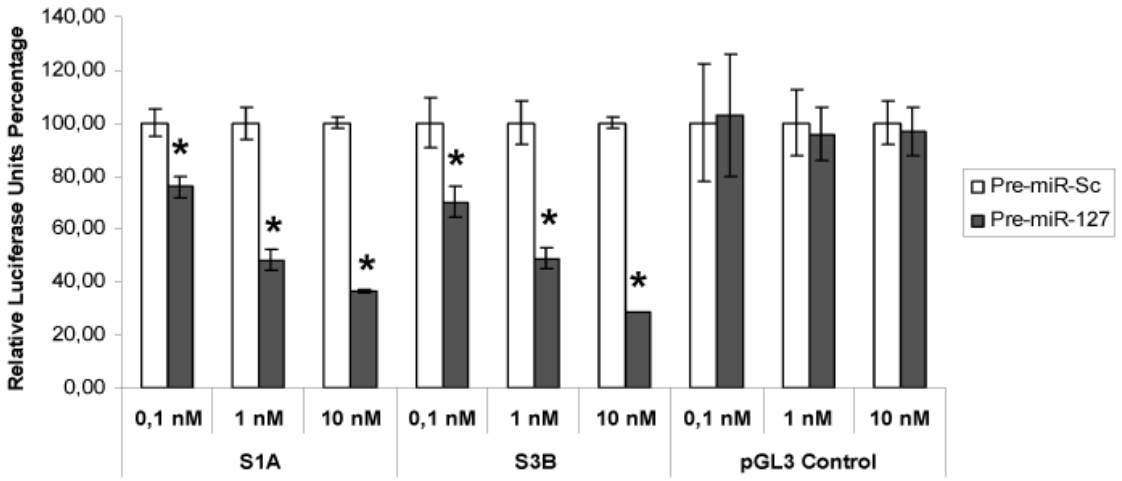

Figure 17: KIF3B is a real target of miR-127 in NRK-52E cells. Luciferase assays of KIF3B 3´UTR vectors, named as S1A and S3B, and empty vector (pGL3-control). Luciferase activity percentage is represented, using renilla activity as normalization control. Asterisks indicate statistical significance between Pre-miR-scramble and Pre-miR-127 transfected cells in each condition ($P<0.05$).

Finally, as KIF3B has been involved in endocytosis and transport along microtubules in proximal tubule cells (Reed AA. et al., 2010), we wanted to study if modulation of miR-127 could affect cell trafficking in our system. For this purpose, we performed non-receptor mediated endocytosis assays in NRK-52E cells transfected with Pre/Anti-miR-127, mimicking the function of substance reabsorption by proximal tubule cells *in vivo*. In these assays, Dextran-FITC is added to culture medium and incubated for 6 hours. After this period, cells are fixed and nuclei are stained with DAPI.

As can be observed in Figure 18A, miR-127 overexpression significantly reduced endocytosis activity, whereas miR-127 blockade markedly rose Dextran-FITC internalization. Quantification of Dextran-FITC internalization confirming these results is shown in Figure 18B. For each image, surface of DAPI signal and green signal were estimated in pixels^2 and quantification was expressed as a Green/DAPI surface ratio.

These results demonstrate that KIF3B is a target gene for rno-miR-127 in NRK-52E cells during H/R, both might be regulating proximal tubule cell trafficking, with relevant implications for renal function.

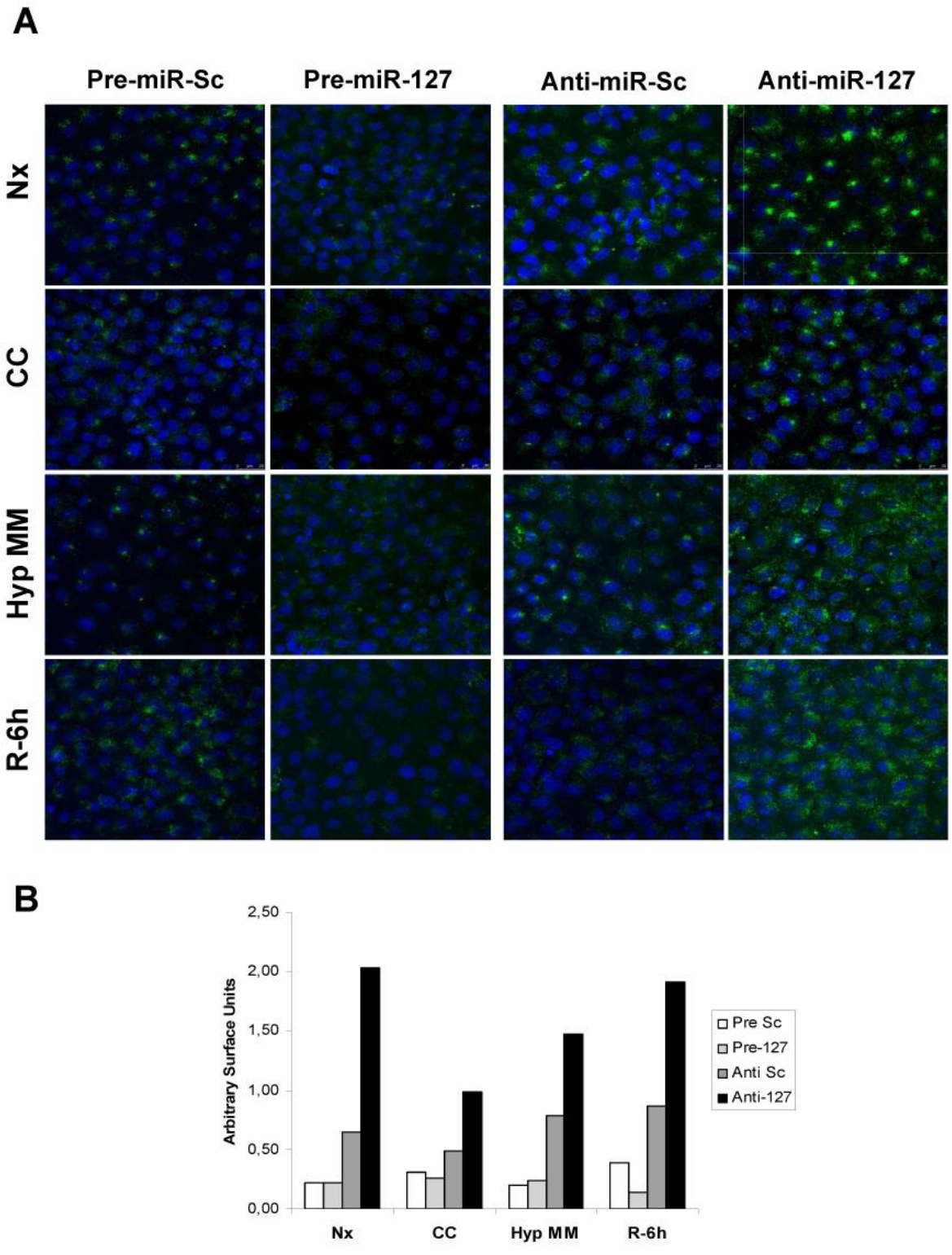

Figure 18: miR-127 regulates trafficking in rat proximal tubule cells in response to H/R. (A) Non receptor-mediated endocytosis assays in NRK-52E cells transfected with pre-miR-127, anti-miR-127 and their respective scramble controls. Dextran-FITC of 70 KDa was added to culture medium and nuclei were stained with DAPI. Representative confocal images from five independent experiments are shown. (B) Quantification of previous images expressed as green signal surface/ blue signal surface ratio, each signal estimated in pixels^2.

5. - Extracellular miRNAs can be detected in supernatants from proximal tubule cell cultures:

As mentioned in previous sections, accumulating evidence during the last five years has demonstrated that miRNAs are present not only inside the cell, but also can be secreted to the extracellular environment, where they exert cell communication and regulatory functions that have not been completely elucidated yet. Based on this evidence, we hypothesized whether proximal tubule cells could secrete miRNAs to the extracellular environment and if these miRNAs could reflect the intracellular miRNA expression events that we have previously observed, becoming biomarkers of cell injury.

To answer this question, we studied hsa-miR-127-3p expression in supernatants from HK-2 cells submitted to H/R. For these experiments, total RNA was extracted following the same procedure detailed for serum samples. Before extraction, supernatants were submitted to preclearing by centrifugation to eliminate cellular debris and avoid false positive results. miR-127-3p was quantified by qRT-PCR using Taqman probes.

As can be observed in Figure 19 miR-127 was successfully detected in supernatants from human proximal tubule cells submitted to H/R, showing a similar expression pattern as observed in previous *in vitro* experiments. These preliminary data clearly demonstrated that proximal tubule cells secrete miRNAs to the extracellular environment, thus these molecules can be biomarkers of proximal tubule responses to several stimuli, including I/R.

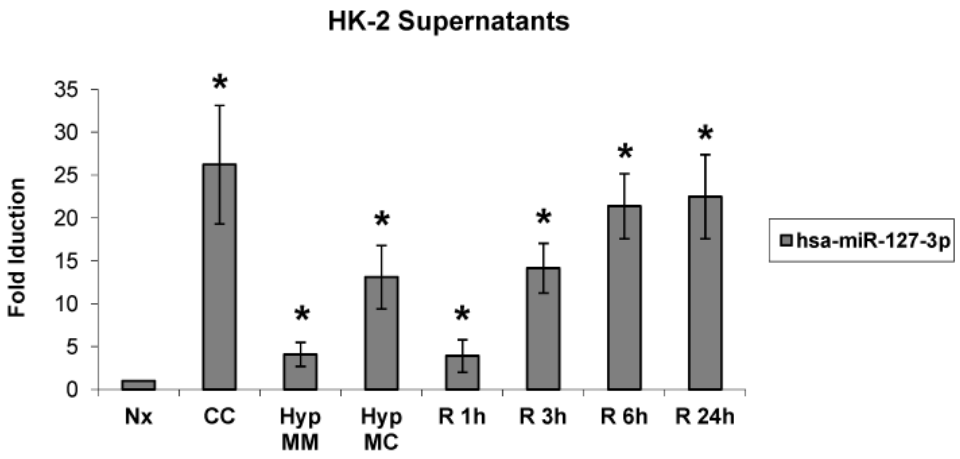

Figure 19: miR-127 is secreted to HK-2 cell cultures supernatants. miR-127 was detected by qRT-PCR in supernatants from HK-2 cultures. Fold changes were calculated using Normoxia (Nx) as reference condition. Data are presented as mean ± s.e.m. of three independent experiments. Asterisks indicate statistical significance comparing each sample with Normoxia (P<0.05).

6. - Serum miRNAs profile is different between healthy people and AKI patients:

Based on this previous evidence, we hypothesized whether miRNAs detected in peripheral body fluids could reflect kidney responses to injury or renal pathologies such as AKI.

To perform these experiments, both serum and urine were considered since these peripheral fluids are easily and routinely obtained in clinical practice. However, we finally chose serum for further studies because anuria is a common feature in AKI, especially in critically ill patients, making difficult to perform studies in this fluid.

To identify seum miRNAs diferentially expressed between healthy people and AKI patients, we performed a massive screening experiment using qRT-PCR arrays. For this initial experiment, two pool of healthy people (n=5 each) and 4 AKI patients were included. In AKI patients, two samples were used, one obtained at time of diagnosis (Day 0) and other at resolution, when serum creatinine values have returned to normal. Clinical settings for these patients can be found in detail in Table 3.

Table 3: Clinical settings of AKI patients and healthy control pools used in the massive screening experiment.

	Age	Gender	AKI Etiologies	AKIN Grade	Serum Creatinine (mg/dl)	
					Diagnosis	Recovery
Healthy Control						
Pool 1 (n=5)	32.8 ± 5.8	2 Men/3 Women	N/A	N/A	0.85 ± 0.12	N/A
Pool 2 (n=5)	36.6 ±8.6	3 Men/2 Women	N/A	N/A	0.74 ± 0.13	N/A
AKI Patients						
1	41	Man	Ischemic	3	13	2.42
2	45	Woman	Toxic	3	4.81	0.68
3	63	Woman	Septic	3	1.53	-
4	47	Man	Ischemic	2	5.01	1.03

N/A: Not Applicable

Raw Cp data was obtained and hierarchical clustering analysis was performed using all the expressed miRNAs. As can be observed in Figure 20, serum miRNA profile successfully classifies the samples included in the experiment. AKI patients cluster apart from healthy controls and toxic recovered sample. Ischemic patients samples are grouped together resulting in two small groups: day 0 (Diagnosis) and day of recovery. Samples from recovered patients are not classified in the healthy

control pool group, probably indicating that, although creatinine levels are normalized and AKI is clinically resolved, there could be an underlying cellular and tissue damage that modifies miRNA expression. By contrast, saple from toxic recovered AKI appears similar to control pools, probably due to the fact that this sample was obtained 2 months after diagnosis. On the other hand, toxic and septic AKI samples show a great distance between them and with ischemic AKI samples. This feature reflects the great biological and molecular differences underlying the different AKI etiologies.

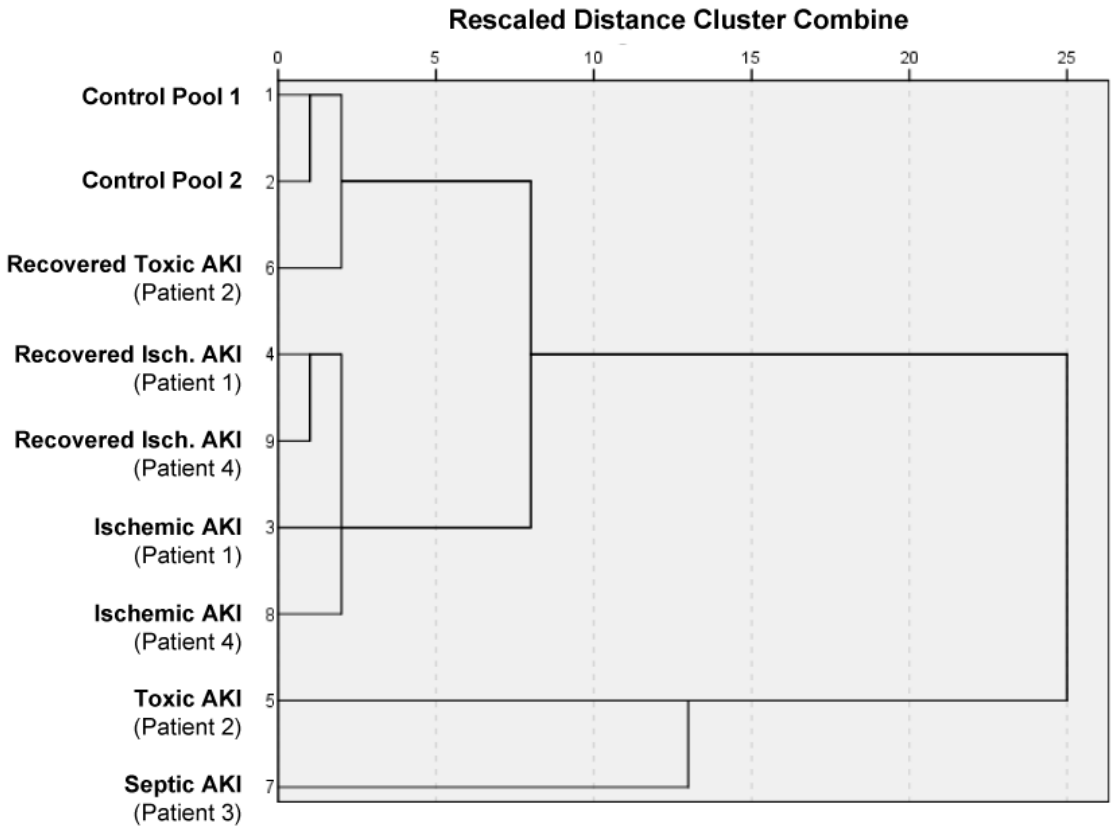

Figure 20: Serum miRNA profile discriminates AKI patients from healthy controls. Hierarchical clustering analysis was performed using raw Cp data from all miRNAs expressed in at least one sample. Obtained dendrogram showing the arrangement of clusters derived from the analysis is shown.

Taken together, all these data demonstrated that serum miRNAs profile can differentiate between AKI patients and healthy people. Moreover, serum miRNAs seems to distinguish AKI etiologies and different recovery degrees over time.

After this initial study, raw expression data was normalized and fold expression changes were calculated using healthy control pools for normalization. Statistical analysis was performed and only miRNAs showing significant changes higher than two folds were considered. For these miRNAs,

bioinformatics target prediction and functional classification was performed using several oline databases. Briefly, for each miRNA a list of potential targets was downloaded from Targetscan 5.1. Next, target genes of each miRNA were analyzed using DAVID database and clustered in functional categories. Statistical analysis was performed for each cathegory to study

relative enrichment compared to human genome background. miRNAs showing targets with biological significance in our study context were taken into account (See material and methods section and Figure 7 for detailed description of selection process).

An integrated study of expression data, statistical analysis and bioinformatics functional studies led to a selection of a panel of 12 miRNAs with potential use as AKI biomarkers. The panel of miRNAs and a representative selection of their potential biological and functional relevance in our study context, as criteria for selection, are summarized in Tables 4 and 5.

Table 4: miRNAs to be tested as AKI biomarkers selected from the massive screening experiment. P-values were calculated comparing miRNA expression in samples obtained at day of diagnosis (Day 0) of AKI patients with healthy control pools.

	Fold Change	p-value
AKI Biomarkers		
hsa-miR-**101-1**	-2.75	0.061
hsa-miR-**127-3p**	-1.98	0.08
hsa-miR-**210**	Undetermined	-
hsa-miR-**126**	-5.75	0.036
hsa-miR-**26b**	-7.52	0.024
hsa-miR-**29a**	-6.62	0.031
hsa-miR-**454**	-5.81	0.015
hsa-miR-**146a**	-8.50	0.021
Ischemic AKI		
hsa-miR-**27a**	-5.09	0.001
Ischemic AKI Recovery		
hsa-miR-**93***	-22.03	0.017
hsa-miR-**487a**	-6.06	0.003
Toxic and Septic AKI		
hsa-miR-**10a**	32.21	0.001

This final panel included different groups of microRNAs which were selected as potential biomarkers of several aspects of AKI. hsa-miR-126, hsa-miR-26b, hsa-miR-29a, hsa-miR-454 and hsa-miR-146a were selected as AKI biomarkers independently of its etiology. These miRNAs showed significant downregulation in AKI day 0 samples compared to control pools. hsa-miR-27a was selected as Ischemic AKI biomarker since this miRNA was downregulated in ischemic AKI patients but did not change its expression in samples from other etiologies. hsa-miR-93* and hsa-miR-487a drastically decreased their expression in ischemic AKI, but they reached healthy-like levels in recovered samples. Thus, these miRNAs were selected as ischemic AKI recovery markers. Finally hsa-miR-10a was selected as septic-toxic AKI biomarker. This miRNA was overexpressed in day 0 samples of patients with AKI of these etiologies. Moreover, bibliography data indicated that hsa-miR-10a is highly and specifically expressed in renal tissue (Landgraf P. et al., 2007).

As can be observed in Table 4 hsa-miR-127-3p, hsa-miR-101-1 and hsa-miR-210 were included in the panel of miRNAs as AKI biomarkers, although they did not show significant changes or they were not detected in the massive screening experiment. miR-127-3p was included based on our data previously obtained in our *in vitro* and *in vivo* experimental models. miR-101-1 was also included because this miRNA was modulated in the initial microarray experiment performed in our *in vitro* model of H/R in NRK-52E cells and previous work of our group indicate that this miRNA is regulated by nutrient depletion in our system (data not shown). On the other hand, hsa-miR-210 was also included because increasing evidence demostrates that this miRNA is universally regulated by hypoxia (Rocha S., 2007) and, as indicated in previous sections of this work, hypoxia is a key component of AKI pathophysiology.

After this initial selection experiment, this panel of miRNAs were validated in larger cohorts of patients, including a cohort of ICU patients with AKI and healthy controls and a cohort of patients who underwent cardiac surgery with CPB.

Table 5: Functional classification of the putative targets of the miRNAs selected as potential AKI biomarkers. Functional categories showed in this table, clustering miRNAs targets, are extracted from KEGG and GO databases.

microRNA	Functional Category	p-value
hsa-miR-101-1	Kidney development	8×10^{-5}
	Cell adhesion	1×10^{-3}
	Endocytosis	1.6×10^{-3}
hsa-miR-127-3p	Ionic channel	1.4×10^{-4}
	Cell adhesion molecules	7.8×10^{-2}
	Epithelium development	$7.9\ \ 10^{-2}$
hsa-miR-210	Basement membrane	1.2×10^{-2}
	Actin cytoskeleton organization	4.1×10^{-2}
	Tube development	6.4×10^{-2}
hsa-miR-126	Kidney development	2×10^{-2}
	Microtubule-based transport	1.3×10^{-2}
	Cell-cell adherent junction	3.5×10^{-3}
hsa-miR-26b	Epithelial tube morphogenesis	7.5×10^{-3}
	ATP binding	8.8×10^{-5}
	Cytoskeleton	7.6×10^{-3}
hsa-miR-29a	Extracellular matrix	8.4×10^{-16}
	Focal adhesion	6.9×10^{-11}
	Collagen type IV	6.5×10^{-7}
hsa-miR-454	Tube development	1.3×10^{-7}
	Endocytosis	2.1×10^{-6}
	Vesicle mediated transport	3.5×10^{-5}
hsa-miR-146a	Microtubule cytoskeleton	1.5×10^{-8}
	Intracellular transport	5.6×10^{-8}
	Kidney development	2.7×10^{-5}
hsa-miR-27a	Kidney development	3.2×10^{-4}
	Endocytic activity	1×10^{-3}
	Epithelial tube morphogenesis	1.9×10^{-3}
hsa-miR-93*	Endocytosis	3.7×10^{-7}
	GTPase regulator activity	1.1×10^{-6}
	Tube development	2.2×10^{-2}
hsa-miR-487a	Tight junction	2.8×10^{-3}
	Intracellular trafficking and secretion	2.9×10^{-3}
	Adherent junction	4.7×10^{-2}
hsa-miR-10a	Endocytosis	2.2×10^{-8}
	Adherent junction	3.7×10^{-8}
	Focal adhesion	1.1×10^{-6}

7. - Serum miRNAs are accurate Biomarkers of AKI in ICU patients:

In a first validation step, we studied the expression of the panel of miRNAs in a cohort of ICU patients with AKI compared to healthy controls. For this study, 35 ICU patients (19 of them presenting ATN and 16 with pre-renal AKI) and 20 healthy controls were selected from two collections stored in Hospital Ramón y Cajal-IRYCIS Biobank. Relevant clinical settings for these samples are presented in Table 6.

Table 6: Clinical settings of ICU patient cohort and healthy controls.

	Healthy Control (n=20)	AKI Patients (n=35)
Age (years)	38.35 ± 11.42	64.9 ± 15.5
Male (n, %)	12, 60%	28, 80%
Female (n, %)	8, 40%	7, 20%
AKI Etiology		
Ischemic	N/A	9
Toxic	N/A	7
Septic	N/A	19
AKIN Grade (Patient number)		
1	N/A	11
2	N/A	9
3	N/A	15
Serum Creatinine (mg/dl)		
Day 0 (Diagnose)	0.76 ± 0.16	2.73 ± 4.47
Day 7 (Recovery)	N/A	1.35 ± 0.98
AKI Origin		
Pre-renal	N/A	16
ATN	N/A	19
Prognostic Score		
SOFA	N/A	6.37 ± 3.68
N/A: Not Applicable		

miRNAs were quantified by qRT-PCR with LNA probes and expression data are presented as ΔCp. For normalization, only Spike-In RNA values were considered because this synthetic oligo, added during RNA extraction, was the most stable and reliable housekeeping, presenting no significant changes between healthy controls and AKI patients. We chose this method for data presentation to show all the control expression values individually, avoiding their normalization to an artificial value. It is important to notice that a unit difference in ΔCt values represents two fold change in the amount of

input miRNA. Absolute differences between both samples can be calculated as $2^{\Delta Cp}$. Higher values of ΔCp indicate lower input amount of the studied miRNA.

7.1. - Serum miRNAs as Diagnostic Biomarkers:

Nearly all the selected miRNAs were successfully detected in serum samples. Only hsa-miR-454 and hsa-miR-487a could not be amplified, resulting in false positives derived from the screening method. Figure 21A shows expression values of selected miRNAs in Day 0 samples (Diagnosis).

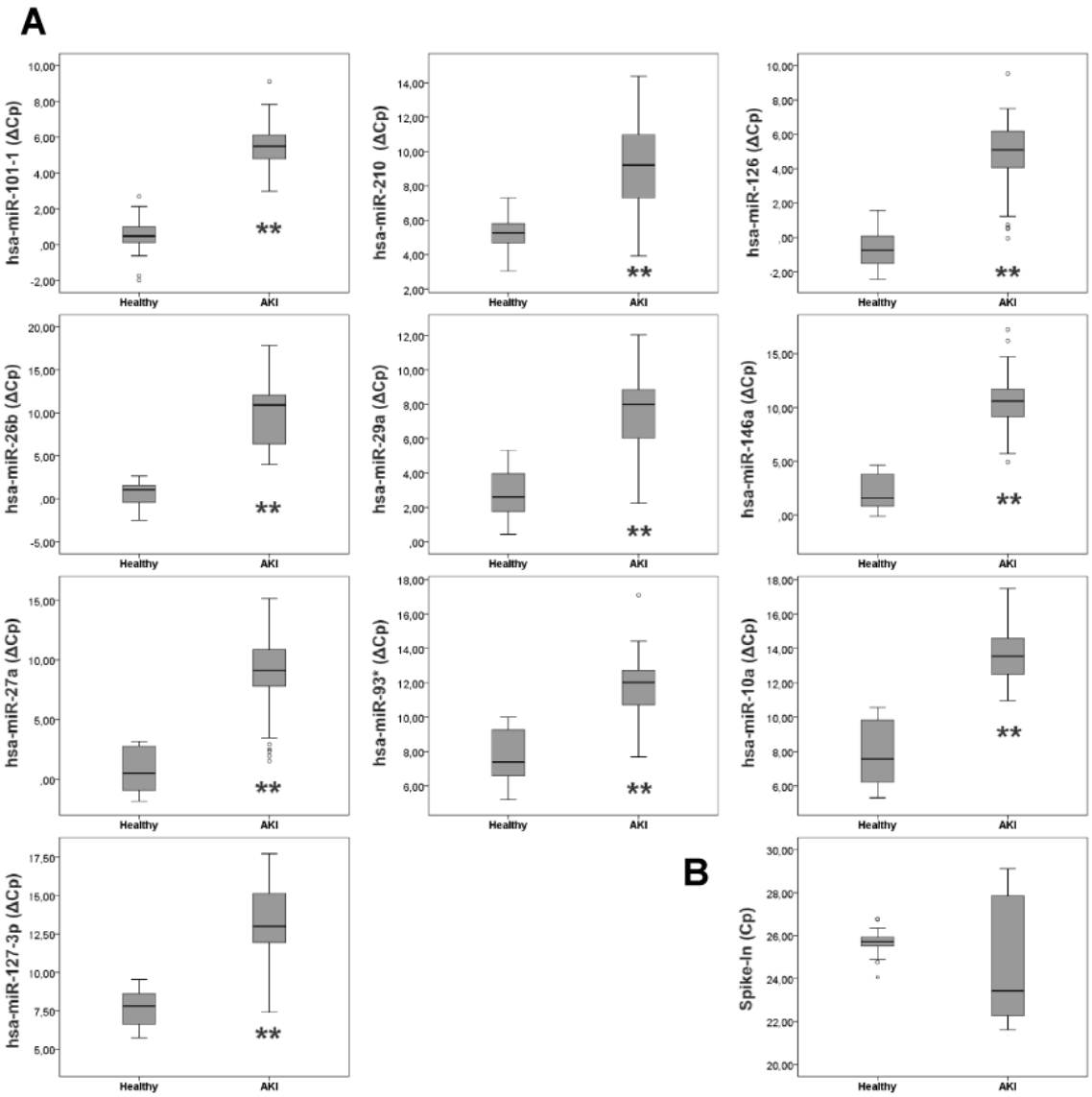

Figure 21: Serum microRNAs are diagnostic biomarkers of AKI in a cohort of ICU patients. (A) miRNAs were detected by qRT-PCR in serum samples from ICU patients at the moment of AKI diagnosis and compared to a cohort of healthy controls. miRNA serum levels are expressed as ΔCp values and data are presented as median and interquartile range. Asterisk indicate statistical significance (*P<0.05 and ** P<0.01) (B) Spike-In raw Cp values demonstrating no significant changes between AKI patients and healthy controls.

In agreement with data obtained in the screening experiment, all the selected miRNA were markedly downregulated (higher ΔCp values) in AKI samples compared to healthy controls. Panel B shows raw Cp values of Spike-In RNA in these samples, showing no statistical significance between healthy and AKI samples (p-value= 0.081).

ROC curve analysis was performed to study the sensitivity and specificity of these miRNAs as diagnostic tools for AKI. As can be observed in Figure 22 and Table 7, all miRNAs presented area under the curve (AUC) values between 0.9 and 1.

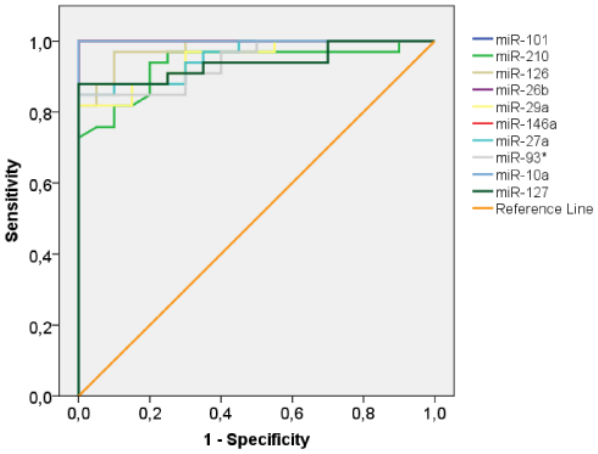

Figure 22: Serum miRNAs show high diagnostic performance in ICU patients. ROC curve analysis of serum levels of the miRNAs included in our set discriminating AKI patients from healthy controls.

Table 7: ROC Curve analysis of ICU patients in the moment of AKI diagnosis compared to healthy control.

microRNA	ROC Analysis Day 0 (Diagnosis)		
	AUC	S.E.M.	p-value
miR-101-1	*1.000*	*0.000*	*0.000*
miR-210	*0.935*	*0.034*	*0.000*
miR-126	*0.979*	*0.015*	*0.000*
miR-26b	*1.000*	*0.000*	*0.000*
miR-29a	*0.948*	*0.27*	*0.000*
miR-146a	*1.000*	*0.000*	*0.000*
miR-27a	*0.955*	*0.025*	*0.000*
miR-93*	*0.942*	*0.029*	*0.000*
miR-10a	*1.000*	*0.000*	*0.000*
miR-127-3p	*0.939*	*0.033*	*0.000*

These data demonstrate that serum miRNAs are a powerful diagnostic tool for AKI with almost 100% of sensitivity and specificity.

7.2. - Serum miRNAs levels correlate with AKI severity:

At the moment, AKI severity is determined based on changes in serum creatinine or urine output produced during a determined time-period. This method delays patient classification with potential dramatic consequences in patient management. Based on this situation, we wanted to assess if serum miRNAs could determine AKI severity earlier than the current diagnostic tools.

As can be observed in Figure 23, Spearman correlation analysis demonstrated that ΔCp values of miR-210, miR-126, miR-29a and miR-146a significantly correlate with patient classification based on AKIN criteria (miR-210 p-value=0.001; miR-126 p-value=0.021; miR-29a p-value=0.009; miR146a p-value=0.008).

These results demonstrate that serum miRNA levels could indicate AKI severity in a single determination avoiding patient classification delaying produced by systems based on creatinine relative changes.

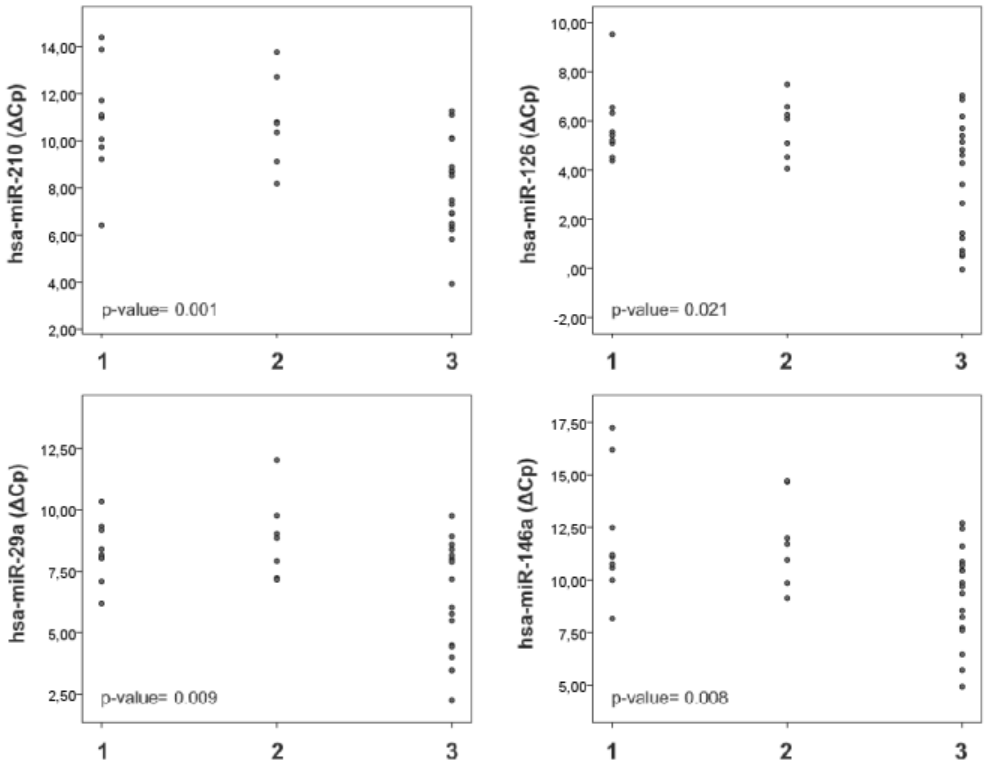

Figure 23: Serum levels of miR-210, miR-126, miR-29a and miR-146a correlates with AKI severity. Serum miRNAs were detected by qRT-PCR in samples from ICU patients at the moment of AKI diagnosis. miRNA expression levels are presented as ΔCp values and each patient is represented by an individual dot. Spearman correlation was performed for each miRNA and p-values are shown in the corresponding plots.

7.3. - Serum miRNAs distinguish between Intrinsic and Pre-renal AKI:

As indicated in previous section of this work, AKI could present an intrinsic origin, when kidney damage is located in renal parenchyma, or pre-renal origin when the kidney cannot function properly due to poor perfusion (Liaño F et al., 2011; Nejat M. et al., 2012). Discrimination between both AKI origins to apply early and appropriate treatment would be a valuable characteristic for an AKI biomarker.

Based on this clinical need, we wanted to assess whether our panel of miRNAs could differentiate between intrinsic and pre-renal AKI. For this purpose, we studied the expression of serum miRNAs in our cohort of ICU patients, comparing ΔCp values between those patients with pre-renal AKI and those with intrinsic AKI. Pre-renal AKI was diagnosed when AKI was recovered in less than 72 hours.

As can be observed in Figure 24, miR-210, miR-126, miR-29a and miR-146a present significant higher ΔCp values in those patients with pre-renal AKI compared to intrinsic AKI indicating the serum levels of these miRNAs could be used to differentiate and diagnose AKI origin.

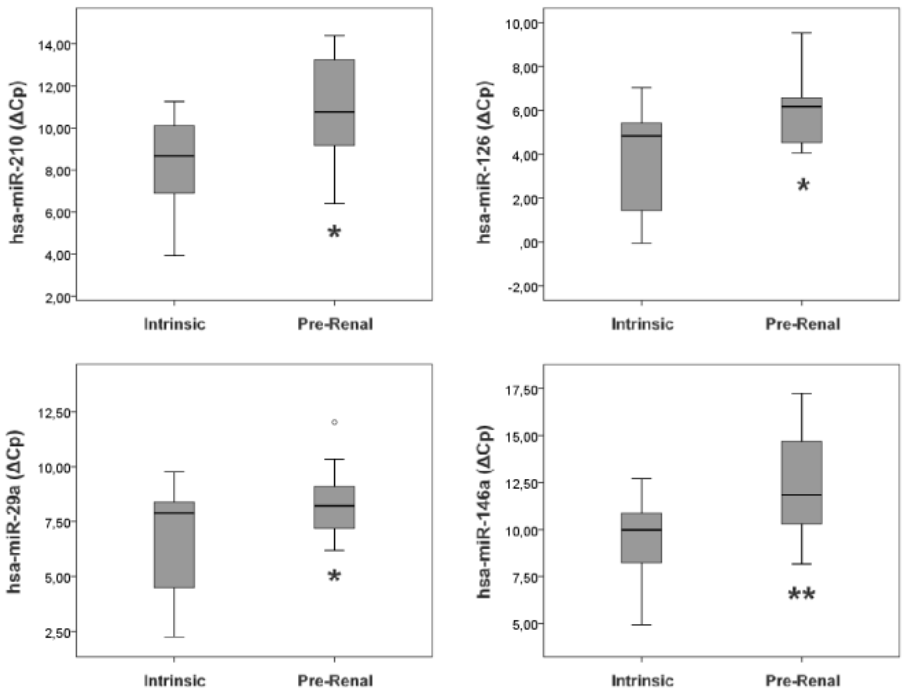

Figure 24: Serum levels of miR-210, miR-126, miR-29a and miR-146a differentiate Pre-renal from Intrinsic AKI. Serum miRNAs were detected by qRT-PCR in samples from ICU patients at the moment of AKI diagnosis. miRNA expression levels are presented as ΔCp values and data are expressed as median and interquartile range. Asterisks indicate statistical significance (*P<0.05 and ** P<0.01).

7.4. - Serum miRNAs as potential biomarkers of long-term outcome:

We studied the expression of our panel of miRNAs in serum samples obtained 7 days after AKI diagnosis, time-point when serum creatinine values are almost recovered (Table 6), indicating that renal function is restored and AKI is clinically resolved.

As can be observed in Figure 25A, the selected miRNAs still shown downregulated values in AKI patients compared to healthy controls. These data agree with the results obtained in the screening experiment, and they could indicate that, although creatinine values are restored, it is possible that there is still a tissue and cellular damage that could be pointed out by miRNAs. Raw Cp data of Spike-In RNA (Figure 25B) show no significant changes between patients and healthy controls.

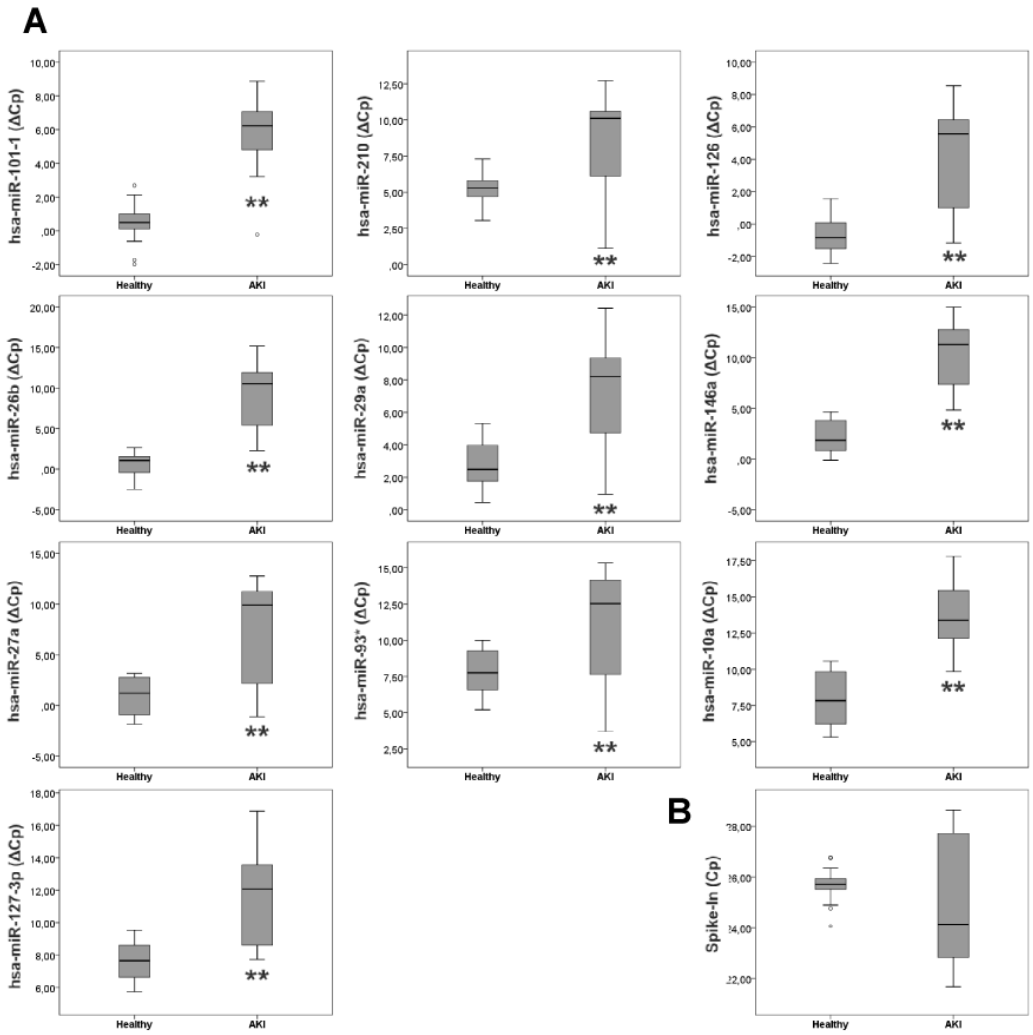

Figure 25: Serum miRNAs levels remain altered when AKI is clinically resolved. (A) miRNAs were detected by qRT-PCR in serum samples from ICU patients obtained at 7 days of evolution and compared to a cohort of healthy controls. miRNA serum levels are expressed as ΔCp values and data are presented as median and interquartile range. Asterisks indicate statistical significance (*P<0.05 and ** P<0.01) (B) Spike-In raw Cp values demonstrating no significant changes between AKI patients and healthy controls.

All miRNAs present AUC values between 0.8 and 1 in ROC analysis (Figure 26, Table 8). Remarkably, miR-146a still presents a 100% specificity and sensitivity.

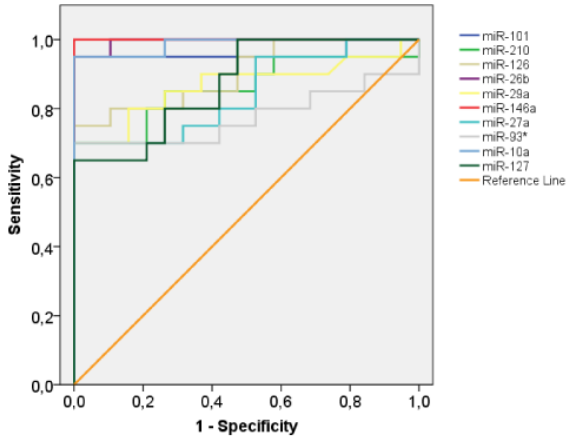

Figure 26: Serum miRNAs levels show high discriminative value between healthy controls and AKI patients at 7 days of evolution. ROC curve analysis of serum levels of the miRNAs discriminating AKI patients at 7 days of evolution after diagnosis from healthy controls.

Table 8: ROC Curve analysis of ICU patients at 7 days of evolution compared to healthy controls.

microRNA	ROC Analysis Day 7 (Recovery)		
	AUC	S.E.M.	p-value
miR-101-1	*0.961*	*0.039*	*0.000*
miR-210	*0.861*	*0.064*	*0.000*
miR-126	*0.903*	*0.049*	*0.000*
miR-26b	*0.995*	*0.007*	*0.000*
miR-29a	*0.867*	*0.063*	*0.000*
miR-146a	*1.000*	*0.000*	*0.000*
miR-27a	*0.845*	*0.064*	*0.000*
miR-93*	*0.776*	*0.084*	*0.003*
miR-10a	*0.987*	*0.015*	*0.000*
miR-127-3p	*0.874*	*0.054*	*0.000*

Taken together all these data demonstrate that the panel of selected miRNAs are accurate biomarkers of AKI in ICU patients, showing high sensitivity and specificity for diagnosis. Moreover, these biomarkers can differentiate between intrinsic and pre-renal AKI, and they show a significant correlation with renal damage degree. miRNA expression values at day 7 remains altered instead of normalization, which could reflect the persistence of cellular and tissue damage. This feature suggests that these molecules could constitute a mark of renal injury when renal function is aparently restored by classical parameters.

8. - Serum miRNAs as biomarkers of AKI after cardiac surgery:

Sublethal AKI is one of the most frequent complications after cardiac surgery (36% Incidence) and its severity is determinant for patient outcome (Liaño F et al., 2011). Due to this clinical relevance, we hypothesized if our panel of miRNAs could predict AKI development in patients undergoing cardiac surgery with cardiopulmonary bypass.

For these experiments, serum samples were collected from a cohort of patients including adults with low and high risk of AKI development as well as pediatric patients. Clinical settings for these patients are summarized in Table 9.

Table 9: Clinical settings of cardiac surgery patient cohort.

	Group IA (n=10)	Group IB (n=10)	Group II (n=15)	Group III (n=4)
Age (years)	65.6 ± 10.4	52.3 (10.6-108.4)*	74.8 ± 6.9	62.0 ± 12.3
Gender (Men/Women)	5/5	7/3	4/10	5/2
BMI (kg/m^2)	27.5 ±3.6	14.5 ± 2.3	26.7 ± 3.7	28.1 ± 2.7
CPB Time (min)	96.6 ± 31.7	58.1 ± 20.3	101.7 ± 34.7	105.2 ± 33.6
ICU L.O.S. (days)	3 (2 – 6)*	5 (3-5)*	6.5 (2 – 10)*	7 (2 – 12.5)*
Basal Serum Cr. (mg/dl)	0.9 ± 0.1	0.57 ± 0.1	1.2 ± 0.3	0.95 ± 0.3
24h Serum Cr. (mg/dl)	0.8 ± 0.2	0.55 ± 0.1	1.1 ± 0.4	1.02 ± 0.6
Discharge Serum Cr (mg/dl)	0.8 ± 0.2	0.5 ± 0.1	1.19 ± 0.4	1.19 ± 0.6
Risk Scores				
LogEuroSCORE	4.08 ± 3.05	N/A	9.7 ± 4.4	9.8 ± 7.8
Cleveland Score	1.8 ± 0.9	N/A	3.83 ± 1.0	4.25 ± 1.0
SRI Score	1 ± 0.5	N/A	3.45 ± 0.5	3.0 ± 0.5
AKI Development				
RIFLE Cr (n)	1	1	4	3
AKIN Cr. (n)	4	1	9	3
Cr. Kinetics (n)	1	1	7	2

BMI: Body Mass Index
N/A: Not Applicable
LOS: Length of Stay.
Cr: Creatinine.
** Data expressed as* median, *25th – 75th percentiles*

As currently available AKI definitions can lead to quite different patient classification, we determined AKI development using the serum creatinine criteria of the RIFLE, AKIN and creatinine kinetics classifications. miRNA were detected by qRT-PCR using LNA probes and, as indicated in previous sections, miRNA expression levels are expressed as ΔCt values using Spike-In RNA as housekeeping control.

8.1. – Serum miRNAs as early Biomarkers of AKI in cardiac surgery patients:

As indicated in material and methods section, in this cohort several serum samples at predefined time points before and after surgery were obtained for each patient. In contrast with ICU cohort, where patients are included in the study when AKI is already established, this experimental design allows a precise AKI development monitoring, including the features which take place in the days previous to diagnosis.

Based on this possibility, we analyzed the expression of our panel of miRNAs during the days before AKI diagnosis. miRNAs were detected by qRT-PCR and expression levels are indicated as ΔCp values. Spearman Rho correlation coefficient was calculated to find out statistically significant correlations between miRNA expression and time-points before AKI diagnosis.

As previously indicated, AKI was determined by RIFLE, AKIN and Creatinine kinetics criteria. The number of patients who developed AKI in each classification was 9, 17 and 11 respectively. We analyzed the expression levels of the miRNAs in the three days prior to AKI episode. Although a tendency could be observed in all classifications, only AKIN criteria reached statistical significance, probably due to the higher number of cases included.

As can be observed in Figure 27, miR-26b, miR-146a, miR-93* and miR-127-3p show a progressive downregulation of their expression levels during the previous days to AKI diagnosis based on serum creatinine criteria. Remarkably, miR-146a shows a pronounced and significant downregulation during the days prior to AKI. These studies demonstrate that this miRNA is a powerful diagnostic biomarker, correlating with previous results obtained in ICU patients, where miR-146a showed 100% specificity and sensitivity.

Taken together these data demonstrate that serum miRNAs are diagnostic biomarkers of AKI, in agreement with the data previously obtained in ICU patients. Moreover, miRNAs can detect and monitor AKI development several days before serum creatinine, becoming early AKI biomarkers which can contribute to improve patient management and outcome after surgery.

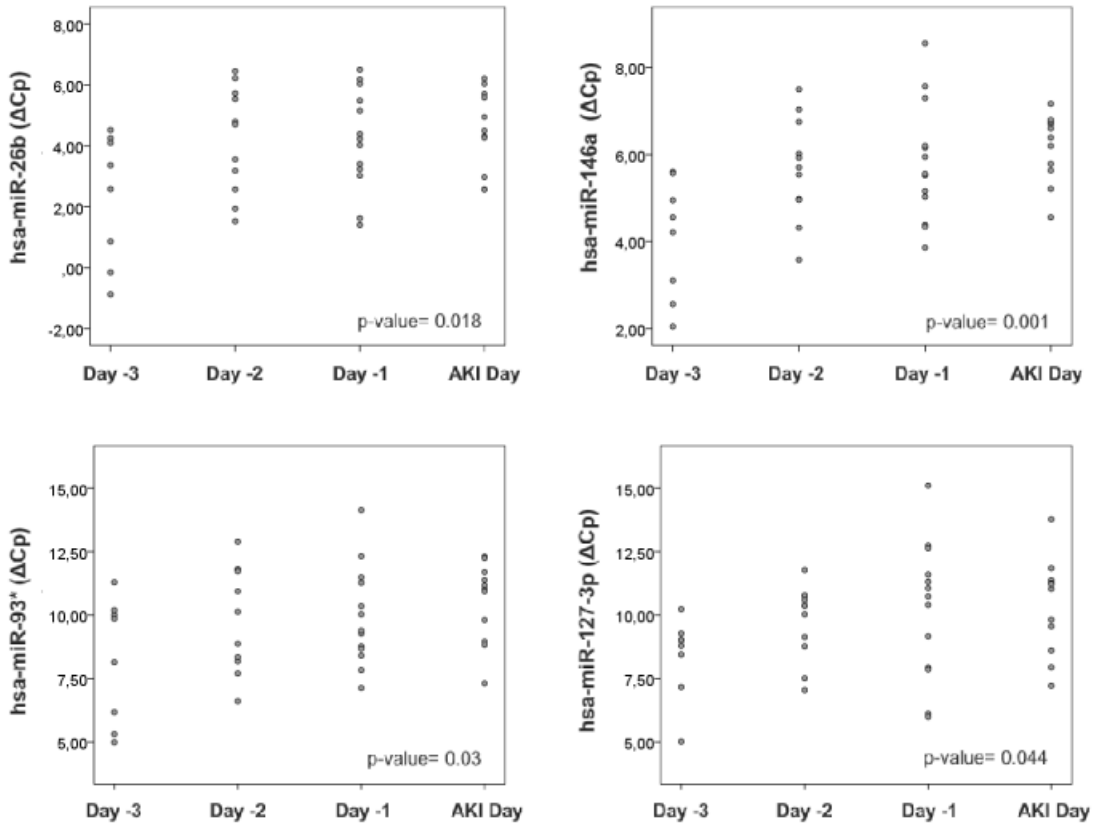

Figure 27: Serum miRNAs downregulation occurs days before serum creatinine increases. miR-26b, miR-146a, miR-93* and miR-127-3p serum levels are expressed as ΔCp values and data is presented as individual dots. AKI was diagnosed based on AKIN serum Cr criteria. Spearman Rho correlation coefficient was calculated and p-values are indicated in each panel.

8.2. - Serum miRNAs are Biomarkers of AKI Predisposition:

In the next step, we wanted to determine whether serum miRNA exhibit differential expression between patients which develop AKI after surgery compared to those who do not develop AKI. For this purpose, all the miRNAs included in our panel were determined in basal serum samples, obtained before surgery. Although some of them presented significant changes depending on the classification used (data not shown), only those miRNAs showing significant changes in all classifications were considered as real biomarkers of AKI development after cardiac surgery.

Spike-In raw Cp values do not significantly change between patients which develop AKI and those which do not develop AKI after surgery in any of the used classifications (RIFLE: p-value=0.450; AKIN: p-value=0.725; Cr Kinetics: p-value=1) (Figure 28A).

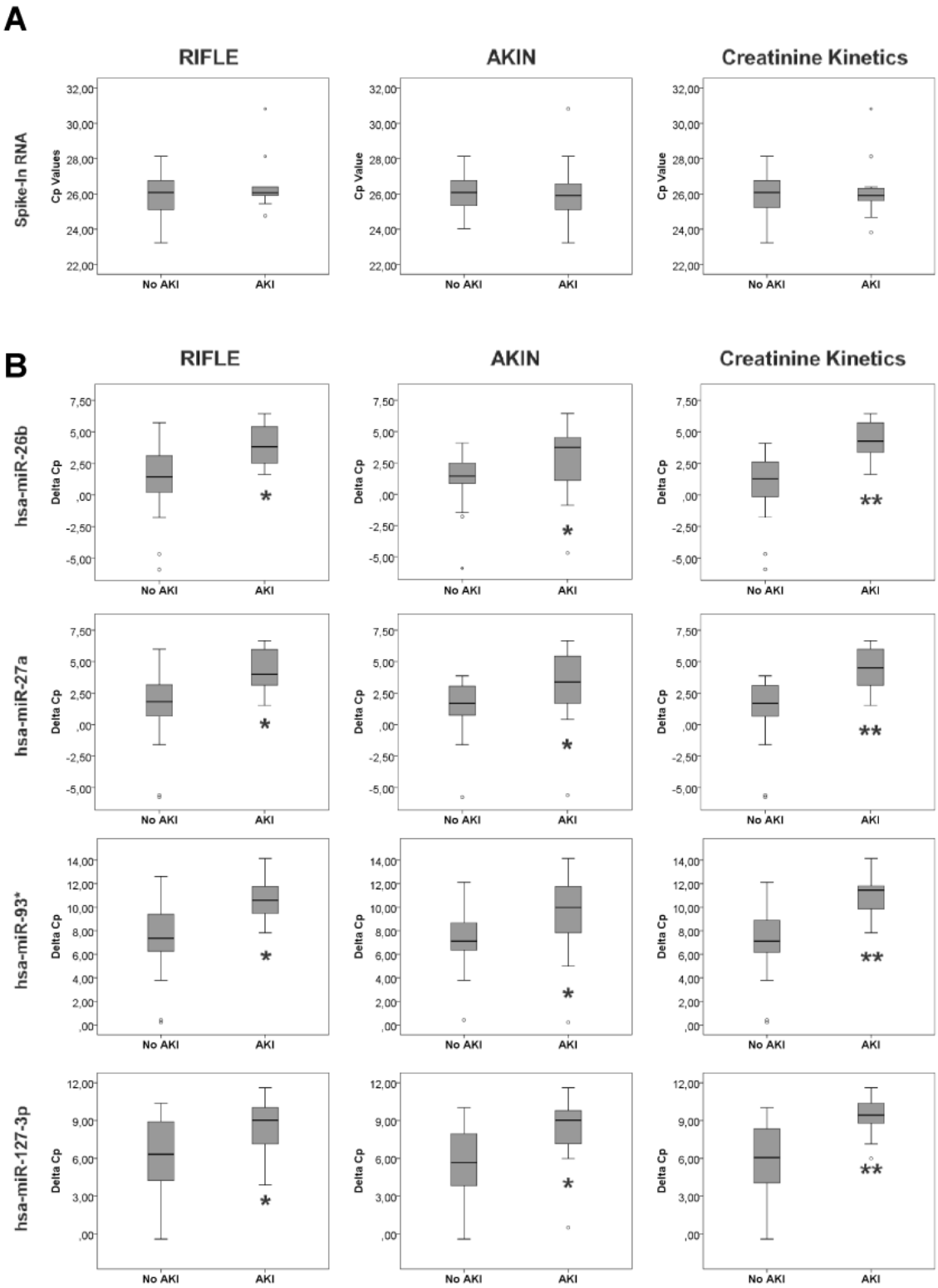

Figure 28: Serum miRNAs estimated before surgery are predictive biomarkers of AKI development. (A) Spike-In raw Cp values are shown indicating no statistical differences between patients who develop AKI compared to those who did not develop AKI after surgery. (B) Serum levels of miR-26b, miR-27a, miR-93* and miR-127-3p were detected by qRT-PCR in serum samples obtained before surgery. miRNA serum levels are expressed as ΔCp values and data are presented as median and interquartile range. Asterisks indicate statistical significance (*P<0.05 and ** P<0.01).

As can be observed in figure 28B, miR-26b, miR-27a, miR-93* and miR-127-3p are significantly downregulated (higher ΔCp values) in those patients who develop AKI after surgery, compared to those who do not develop AKI. Interestingly, those miRNAs which were selected as biomarkers of ischemic AKI (Table 4) are significantly regulated in this system, in agreement with the data obtained in the initial screening experiment.

ROC analysis was performed to study the diagnostic potential of these miRNAs as predictors of AKI. As it is shown in Figure 29 and Table 10, all the studied miRNAs present significant AUC values between 0.7-0.9, depending on the AKI definition criteria used, demonstrating high diagnostic value.

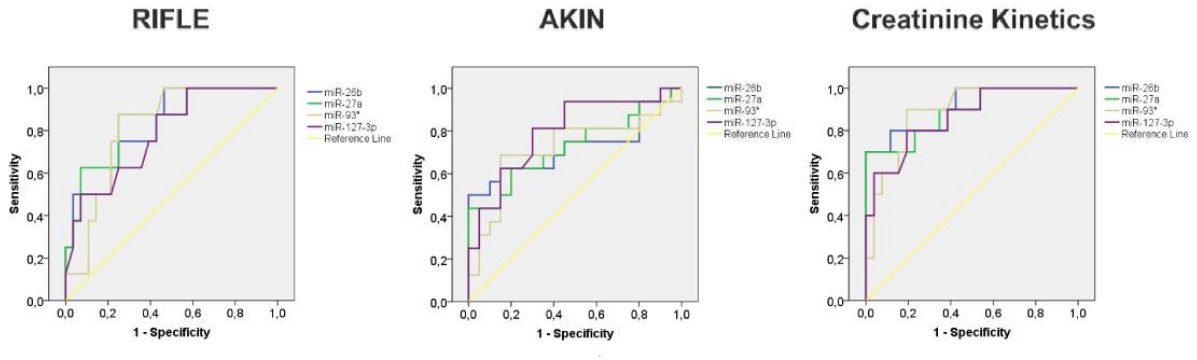

Figure 29: Serum miRNAs levels indicate AKI predisposition. ROC curve analysis of serum levels of miR-26b, miR-27a, miR-93* and miR-127-3p discriminating between patients who develop AKI and those who do not develop AKI after surgery.

Table 10: ROC Curve analysis of serum miRNAs estimated in samples obtained before surgery.

	RIFLE		AKIN		Creatinine Kinetics	
	AUC	**p-value**	**AUC**	**p-value**	**AUC**	**p-value**
miR-26b	*0.821*	*0.006*	*0.722*	*0.024*	*0.908*	*0.000*
miR-27a	*0.844*	*0.007*	*0.725*	*0.022*	*0.888*	*0.000*
miR-93*	*0.815*	*0.007*	*0.719*	*0.026*	*0.887*	*0.000*
miR-127-3p	*0.783*	*0.016*	*0.795*	*0.003*	*0.863*	*0.001*

Similar studies were performed with serum samples obtained immediately after surgery. Although all the miRNAs are also downregulated in patients which develop AKI, only miR-27a and miR-93* show significant changes depending of the AKI definition criteria used (Figure 30).

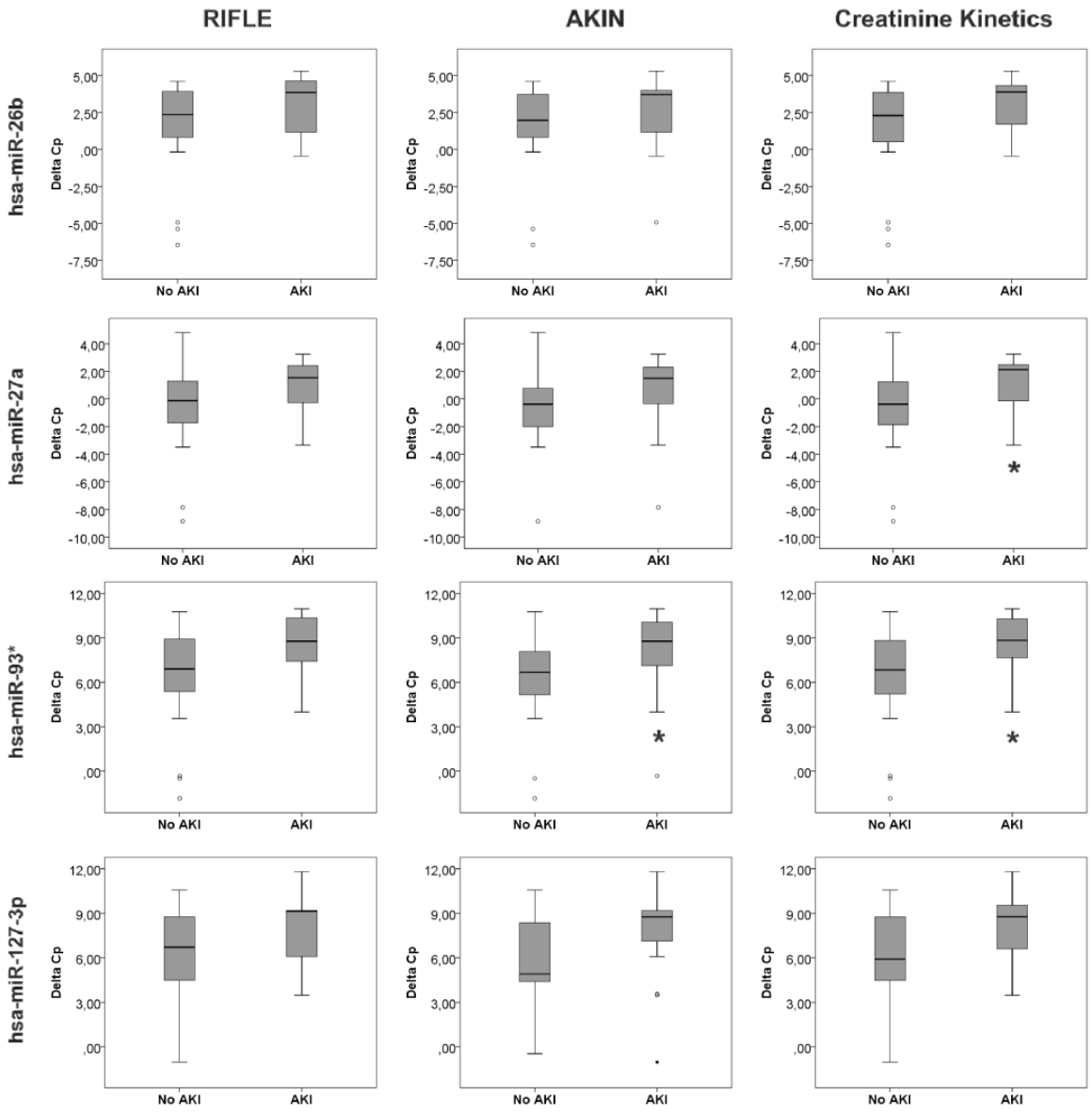

Figure 30: Serum levels of miRNAs estimated immediately after surgery do not discriminate later AKI development. Serum levels of miR-26b, miR-27a, miR-93* and miR-127-3p were detected by qRT-PCR in serum samples obtained immediately after surgery. miRNA serum levels are expressed as ΔCp values and data are presented as median and interquartile range. Asterisks indicate statistical significance (*P<0.05).

ROC analysis of these data indicates lower diagnostic potential with AUC values between 0.6-0.7 (Figure 31, Table 11).

Taken together, these data demonstrate that basal levels of miR-26b, miR-27a, miR-93* and miR-127-3p are promising biomarkers of AKI predisposition, although not significant differences were found after surgery.

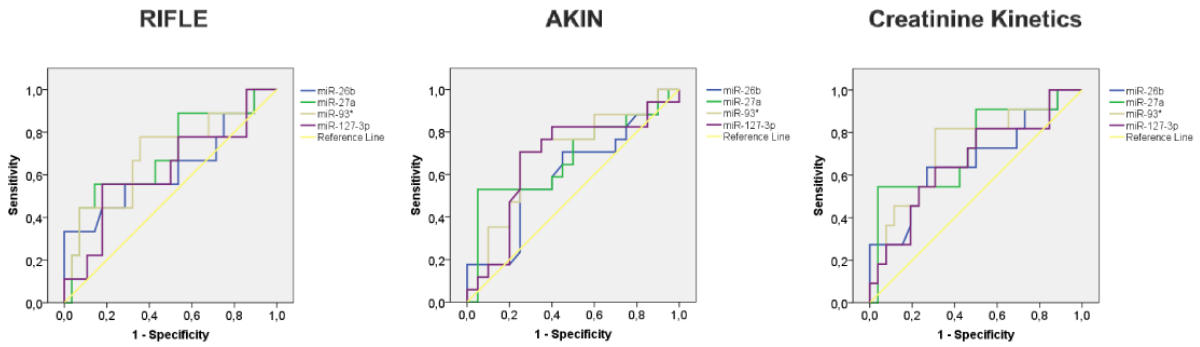

Figure 31: Serum levels of miR-26b, miR-27a, miR-93* and miR-127-3p immediately after surgery do not exhibit high sensitivity and specificity for AKI. Discriminative analysis was performed comparing those patients who develop AKI and those who do not develop AKI after surgery.

Table 11: ROC Curve analysis of serum miRNAs estimated in samples obtained immediately after surgery.

	RIFLE		AKIN		Creatinine Kinetics	
	AUC	**p-value**	**AUC**	**p-value**	**AUC**	**p-value**
miR-26b	*0.629*	*0.250*	*0.601*	*0.293*	*0.66*	*0.114*
miR-27a	*0.694*	*0.083*	*0.676*	*0.067*	*0.727*	*0.031*
miR-93*	*0.698*	*0.077*	*0.697*	*0.041*	*0.731*	*0.028*
miR-127-3p	*0.623*	*0.272*	*0.672*	*0.075*	*0.664*	*0.118*

DISCUSSION

In this work, we have identified and characterized several miRNAs as key molecules involved in kidney response to I/R as well as their role as promising AKI biomarkers. Identification of molecular mechanisms underlying kidney ischemic injury and recovery is essential for improving our understanding of AKI pathophysiology and new therapeutic target identification. Moreover, discovery of earlier and more accurate biomarkers could significantly improve AKI patient management in several clinical contexts, such as ICU, renal transplant or cardiac surgery.

We have identified rno-miR-127 and its human homologous hsa-miR-127-3p as important mediators of the proximal tubule response to I/R. rno-miR-127 induction during I/R is a cytoskeleton protection mechanism which prevents actin depolimerization and promotes cell adhesion by preventing FAC disassembly and TJ disorganization. Moreover, we have identified KIF3B, a component of kinesin II complex (Manning BD and Snyder M, 2000), as a real target of rno-miR-127 in proximal tubule cells, with potential implications in cell trafficking. In addition, we have demonstrated that hsa-miR-127-3p and a panel of serum miRNAs identified in this work (miR-101-1, miR-210, miR-126, miR-26b, miR-29a, miR-146a, miR-27a, miR-93* and miR-10a) are precise biomarkers of AKI in ICU and cardiac surgery patients. Indeed, miR-210, miR-126, miR-29a and miR-146a can discriminate between pre-renal and intrinsic AKI and their expression levels correlate with AKI severity. Moreover, miR-26b, miR-27a, miR-93* and miR-127-3p are biomarkers of AKI predisposition and miR-26b, miR-146a, miR-93* and miR-127-3p indicate kidney damage and AKI development before serum creatinine increases.

Several studies have identified miRNAs modulated during renal I/R injury (Wei Q et al., 2010; Shapiro MD et al., 2011) but none have pointed miR-127 as a regulated miRNA in this context. Within our knowledge, this is the first study identifying and characterizing miR-127 in kidney response to I/R. Previous publications have described miR-127 as an ubiquitously expressed microRNA which can be detected in several human and rat tissues including kidney and proximal tubule cells (Landgraf P et al., 2007; Linsen SE et al., 2010). Aditionally, this microRNA is also expressed in other human epithelial cells such as breast (Bockmeyer CL et al., 2011) and lung (Bhaskaran M et al., 2009)

Our results demonstrated for the first time that miR-127 is regulated by ischemia *in vitro* and *in vivo*. Several microRNAs modulated by hypoxia have been identified (Crosby ME et al., 2009) but miR-127 was not included among them. It is important to notice that our models (*in vitro* and *in vivo*) not only include low oxygen levels, but also serum and nutrient deprivation, which can explain miRNAs different profile between hypoxia and ischemia conditions. In this regard, it has been described that miR-127 is involved in the response of pancreatic cells to glucose availability to produce insulin secretion (Bolmeson C et al., 2011). In any case, our results indicate a great

correlation between the expression of rno-miR-127 in both the *in vitro* and *in vivo* model in rat. rno-miR-127 is increased during hypoxia and Ischemia and at 1h and 24 hours of reperfusion respectively, time points when cellular or renal tissue damage is maximum in this model (Sáenz-Morales D et al., 2006; Conde E et al., 2012). These results indicate that miR-127 could be a potential renal tissue damage biomarker, as we have probed in AKI patients, and it could have a potential role in proximal tubule response to I/R as it will be further discussed.

On the other hand, we observed different miR-127 expression pattern in human cells (HK-2), where its expression is increased mainly during complete medium hypoxia and along reperfusion. Although miR-127 is located in a cluster of microRNAs whose structure is conserved among mammals, miR-127 promoter region shows very low sequence conservation between rat and human. Due to this fact, transcription factor binding sites exclusive for each species could contribute to the observed different regulation (Song G and Wang L, 2009). Moreover, in human cells, hsa-miR-127-3p is located in a CpG island which could be submitted to a fine tissue specific regulation by methylation. In several human tissues and in some types of cancer, DNA hypermethylation leads to repression of miR-127 expression (Lujambio A et al., 2010). Surprisingly, evidence in rat hepatocarcinogenesis models reveals that hypomethylation of DNA, induced by a methyl-deficient diet, decreases miR-127 expression (Tryndyak VP et al., 2009), indicating that although epigenetic DNA regulation can explain differences in miRNAs expression between species, miR-127 regulation by methylation is still controversial.

Our interference experiments *in vitro* demonstrated for the first time that HIF-1α is one of the regulators of miR-127 expression, among other mechanisms. Moreover, HIF-1α is induced during H/R and I/R as we have previously demonstrated (Conde E et al., 2012). A putative HRE element downstream miR-127 sequence, highly conserved among species, was bioinformaticaly predicted. However, CHIP analysis did not confirm HRE functionality. This could indicate that other HRE elements not predicted by the bioinformatic analysis could be responsible for miR-127 regulation in our *in vitro* system. Besides, this regulation by HIF-1α could be indirect. In this regard, a potential crosstalk between c-myc and HIF-1α could be suggested in this case, since c-myc is a known regulator of miR-127 expression in leukemia cells (Leucci E et al., 2010). Additionally, HIF can modulate the expression of histone demethylases involved in chromatin remodeling (Beyer S et al., 2008), which is needed for the expression of some essential genes for the hypoxic response such as EPO (Rocha S, 2007) or miRNAs (Kim GH et al., 2011).

We have identified here for the first time KIF3B as a real target of rno-miR-127 in rat proximal tubule cells during H/R. Proximal tubule cells present a developed endocytosis apparatus

involved in urine protein absorption and membrane receptors exposition and recycling (Brown D et al., 2009). KIF3B has been involved in late endosomes and lysosomes localization (Brown CL et al., 2005). However, recent work has demonstrated that KIF3B is also responsible for receptor and ionic transporter localization in polarized epithelial cell membrane. In this regard, it has been evidenced that KIF3B knockdown impairs cell polarization in intestine epithelial cells (Gao N and Kaestner KH, 2010) and it is important for the correct localization of kidney anion exchanger 1 and Chloride/proton antiporter CLC-5 in proximal tubule cells (Duangtum N et al., 2011; Reed AA et al., 2010). In addition, KIF3B has been unveiled as a specific regulator of albumin and transferring uptake in polarized kidney cells. KIF3B overexpression promotes internalization of membrane vesicles containing cubilin and megalin receptors, decreasing proximal tubule protein reabsorption capacity (Reed AA et al., 2010). In this work, rno-miR-127 blockade leads to KIF3B overexpression and endocytic activity increase. Thus, miR-127 up-regulation observed during I/R could result in KIF3B downregulation and proximal tubule cell trafficking impairment, as observed during renal I/R injury (Bonventre JV and Yang L, 2011). Moreover, miR-127 induction and trafficking impair trough KI3FB inhibition could lead to tubular cell protection since cell trafficking requires high levels of ATP, compromised during renal I/R.

Regarding a potential protective role of miR-127 in response to I/R, this work describes for the first time the effects of rno-miR-127 modulation in actin cytoskeleton organization and adhesive structures integrity during I/R injury. miR-127 overexpression prevents FAC disassembly and TJ disruption and epithelial barrier impairment, all of them essential for kidney function. The molecular mechanisms responsible for these effects need to be further investigated, but the regulation of intestinal TJ permeability by miRNAs has been recently described (Ye D et al., 2011). Other components of cell-cell adhesion structures such as E-cadherin are also regulated in kidney by miRNAs, in particular, miR-200 family (Xiong M et al., 2012). Furthermore, E-cadherin function and AJ integrity could be indirectly regulated by miR-127 target KIF3B. This factor directly interacts with plakophilin-4, which is involved in E-cadherin maintenance in the cell surface and its connection to cytosleketon. Moreover, KIF3B is a regulator of Rho-A activity during stress fibers formation (Apodaca G, 2001), that has been previously observed in proximal tubule cell response to I/R (Sáenz-Morales D et al., 2006). Therefore, rno-miR-127 induction during I/R and H/R could protect cell-matrix and cell-cell adhesions trough KIF3B downregulation, among other mechanisms not yet identified, contributing to cell structure maintenance and epithelial barrier function.

Since miRNAs can regulate injury and repair cellular processes, functional studies of the miRNAs included in our panel of AKI biomarkers may provide additional worthy information for AKI biology understanding. This is the case of miR-127-3p, which has been unveiled in our work as an accurate biomarker of ischemic AKI in cardiac surgery patients and also as a regulator of proximal

tubule cell adhesion and trafficking. In addition, due to their critical role in pathophysiological processes, miRNAs could be considered as new potential therapeutic targets.

hsa-miR-127-3p secretion to supernatants of proximal tubule cell cultures pointed out the potential role of extracellular miRNAs as AKI biomarkers. Our data show a great correlation between extracellular and intracellular expression pattern of miR-127-3p in HK-2 cells submitted to H/R. miRNA secretion mechanisms and regulation are not completely understood yet. Recent evidence indicates that miRNA secretion to extracellular fluids is a selective process and that secreted miRNAs do not reflect the miRNA profile of the parental cells (Mittelbrunn M et al., 2011). However, it has been also demonstrated that some miRNAs, such as let-7 family, exhibit correlating intra- and extracellular levels (Koh W et al., 2010).

miRNAs quantification in serum samples is not standardized yet and several technical questions need to be further optimized. Both serum and plasma have been used for miRNA biomarker discovery leading to extremely different results. Plasma contains a higher concentration of circulating miRNAs. However, these plasma miRNAs principally derive from platelets (McDonald JS. et al., 2011). Based on this evidence, we finally chose serum to perform our studies, since platelet contamination could introduce a great error in miRNA profiling and quantification.

Several protocol variations have been used for serum miRNA retrotranscription after total RNA extraction. Some approaches normalize RNA amount among reactions, while other studies, such as this work, use a constant volume of eluted RNA for this purpose. Due to the extremely low amount of miRNAs contained in 200 µl of serum, RNA quantification by Nanodrop or Bioanalyzer chips is not reliable enough. Moreover, we have not found correlation between RNA concentration, estimated by both methods, and miRNA amplification (data not shown). In addition, constant RNA amount strategy leads to a disproportionate representation of the original volumes of these miRNAs were derived from (Gallo A. et al., 2012). A constant elution volume during RNA extraction and a constant RNA volume for retrotranscription, represent an invariant volume of serum, potentially allowing miRNA concentration calculation using standard curves and absolute quantification strategies.

Data normalization is other of the key points for reliable serum miRNA quantification. Several housekeeping approaches have been proposed such as small nucleolar RNAs, constant miRNAs previously tested in tissue studies, or synthetic Spike-in RNAs (For review Reid G. et al., 2011). In our experience, few small nuclear RNAs could be detected in serum samples and those which could be quantified did not show stable levels across samples. Small ribosomic RNAs, such as 5s, could not be amplified in our system either. Moreover, some of the miRNAs which have been widely used as

housekeeping such as miR-16 or miR-let-7a were modulated in our samples. In addition, several studies point out that the principal source of technical variation in serum miRNA quantification is the RNA extraction step (Andreasen D. et al., 2010; McDonalds JS. et al., 2011). Based on these evidences, we finally chose Spike-In normalization method by adding the RNA oligonucleotides during the first steps of RNA extraction.

In this work, after setting up the protocols as well as quantification and normalization methods, we identified and validate, in a pilot study, a set of miRNAs, including miR-101-1, miR-127-3p, miR-210, miR-126, miR-26b, miR-29a, miR-146a, miR-27a, miR-93* and miR-10a, as potential biomarkers of AKI in ICU and cardiac surgery patients. Genome wide profiling experiments have demonstrated that some of these miRNAs (miR-10a, miR-27a, miR-29a, miR-101 and miR-210) are highly expressed in human kidney tissue (Liu GC et al., 2004; Landgraf P et al., 2007). Indeed, several studies have confirmed the presence of some of these miRNAs in serum microvesicles and miR-101, miR-27, miR10a, miR-146a, miR26b and miR-29a have been specifically detected in microvesicles derived from renal carcinoma cells, with a potential role in tumor angiogenesis regulation (Hu G et al., 2012; Grange C et al., 2011).

Some of these miRNAs have been related to kidney injury in several studies. miR-146a regulates it expression in kidney in mice submitted to renal I/R injury (Godwin et al., 2010). Moreover, increased levels of this miRNA are present in renal tissue and urine of patients with IgG nephropathy. This study also demonstrates that, in agreement with our data, miR-146a levels correlates with the clinical severity of injury, indicating that this miRNA could be a modulator of the pathophysiological mechanisms of kidney injury (Wang G. et al., 2011).

On the other hand, miR-29a has been related to kidney fibrosis regulation and chronic kidney disease progression. This miRNA is regulated by TGF-β and it expression has been related to fibrosis prevention, by avoiding ECM deposition in renal parenchyma (Patel V and Noureddine L V, 2012).

In a recent study of Lorenzen JM. et al., miR-210 expression levels in plasma predicts survival in critically ill patients with AKI. In contrast with our data, in this study AKI patients present higher levels of miR-210 compared to healthy controls, probably due to the use of plasma samples instead of serum. Survival was studied 4 weeks after starting of RRT. miR-210 levels were significantly higher in non-survivor patients compared to survivors (Lorenzen JM. et al., 2011).

A recent work from Saikumar J. et al. has identified miR-21, miR-155 and miR-18a as miRNAs regulated in kidney tissue during AKI. Moreover, urinary levels of miR-21 and miR-155 are increased in AKI patients compared to healthy volunteers (Saikumar J. et al., 2012). Although these miRNAs

were not finally selected in our panel of miRNAs of interest, in agreement with this study, miR-155 and miR-21 were also modulated in our initial screening experiment (data not shown). These data suggest that miRNAs associated with AKI could be modulated in several body fluids, particularly in serum and urine, presenting a potential expression correlation.

In a first step of proof-of-concept studies, our data in ICU patients demonstrate that our panel of miRNAs presents a high diagnostic potential with AUC values between 0.9 and 1 in ROC analysis. This discriminative power is higher than that obtained with other AKI biomarkers currently in development. Plasma and urinary NGAL, which have demonstrated the highest diagnostic potential up to the moment, present AUC values between 0.78 and 0.86 in adult ICU studies. Cystatin C and IL-18 have demonstrated AUC values of 0.7 and 0.62 respectively (For review Sirota JC. et al., 2011). Our results demosnstrate that serum miRNAs show a very high diagnostic value in adult population, compared with other biomarkers which only reach sensitivity and specificity values close to 100% in pediatric ICU populations.

Other characteristic of a valuable AKI biomarker is the ability to achieve an objective and precise quantification or renal injury. Serum levels of miR-210, miR-126, miR-29a and miR-146a show a significant and robust correlation with AKIN stage classification. This feature indicates that these miRNAs could be biomarkers of AKI severity, since alterations in these miRNAs could be underlying AKI development. Quantification of these miRNAs could indicate AKI severity in a single determination, allowing earlier patient classification. Among the wide range of new AKI biomarkers, only serum Cystatine-C present this ability (Soto K. et al., 2010), although others such as plasma NGAL can predict AKI progression in severity (Koyner JL. et al., 2010).

Another goal of the new generation of AKI biomarkers is the ability to differentiate between pre-renal and intrinsic AKI. Clinical decision making is currently non-optimal and too slow with the present diagnostic methodologies. At the moment, pre-renal AKI is treated by vigorous hydration and waiting for kidney functional damage reversal. Waiting for reversal delays diagnosis and appropriate intervention in those patients which require more than rehydration treatment. Thus new diagnostic tools are required instead of diagnosis by exclusion. Several biomarkers have demonstrated ability to detect pre-renal AKI. KIM-1, Cystatin-C and IL-18 increase their concentration in pre-renal patients compared to no-AKI patients. However, these findings are still controversial and several studies indicate that these biomarkers as well as NGAL do not change its concentration in pre-renal patients, showing similar levels to no-AKI patients (Nickolas TM. et al., 2008; Payen D and Legrand M, 2011; Nejat M. et al., 2012).

Our data demonstrate that serum levels of miR-210, miR-126, miR-146a and miR-29a are significantly downregulated in pre-renal patients compared to established AKI patients, indicating that these biomarkers can discern between both AKI origins. Recently, pre-renal AKI has been defined as a mild form of intrinsic AKI which can be reversed, rather than two differents forms of AKI (Nejat M et al., 2012). Interestingly, target genes of these miRNAs include critical genes involved in renal homeostasis and regeneration after injury such as E2F3, target gene of miR-210 involved in cell cycle regulation (Giannakakis A et al., 2008) and Collagen I and IV, miR-29a targets and essential components of renal ECM (Wang B et al., 2012). Additionally, secretion of miR-126 by endothelial cells is involved in reprogramming of hypoxic resident renal cells to a regenerative program (Cantaluppi V et al., 2012).

On the other hand, both initial cluster analysis and ICU patient data when creatinine is normalized demonstrate that serum miRNA profile can distinguish between different recovery degrees and maintain altered levels even when renal function is reestablished. AKI is a complex syndrome which includes not only functional injury but also structural damage. In the last years, accumulating evidence demonstrates that, although the majority of patients present functional recovery after AKI, estimated by serum creatinine, there is a remaining tissue damage. This sub-clinical injury leads to decreased renal reserve and ultimately long-term adverse outcomes such as Chronic Kidney Disease (Murugan R and Kellum JA, 2011). Serum miRNAs could constitute new biomarkers of kidney structural damage indicating underlying tissue injury even when renal function, estimated by classical parameters, is restored. Our data demonstrate that serum miRNAs levels remain downregulated at 7 days of evolution after AKI. In agreement with this feature, it has been recently demonstrated that CKD development is associated with a lower circulating miRNA concentration in serum (Neal CS. et al., 2011).

Due to their key role in regulation of cell function, miRNAs can also constitute a biological mechanism involved in long-term pathology development after AKI. In this regard, maintained hypoxia has been described as an important factor in development of CKD and other renal pathologies (Heyman SN et al., 2008). Thus miRNAs regulated by hypoxia could have important functions in these events. miR-210, one of the miRNAs included in our panel, is one of the most characterized miRNA regulated by hypoxia, with essential cellular adaptative functions (For review Chan YC. et al., 2012). Thus, maintained deregulation of this miRNA, among others, could constitute a mechanism underlying long-term pathology development and serum levels could be used to monitoring and predict patient outcome.

Other important characteristic for an AKI biomarker is the ability to provide information about the etiology of the injury. IL-18 and NGAL present higher levels in septic AKI patients compared to non-septic AKI (Bagshaw SM. et al., 2007; Bagshaw SM. et al., 2010) and they also change their expression levels in contrast and cisplatin-induced AKI (Sirota JC. et al., 2011). However, despite these promising evidences, up to the moment none of them have demonstrated ability to discern AKI etiology in a cohort of mixed patients including ischemic, toxic and septic patients. Our screening data demonstrate that serum miRNA expression profile could distinguish between patients with several AKI etiologies, probably due to the different biological mechanism underlying each one of them. Thus, a careful selection of specific miRNAs for each etiology could undoubtedly indicate AKI etiology in a single determination, with potential application in complex cohorts of mixed patients. However, this hypothesis needs to be further investigated.

Early AKI detection before functional changes are detectable is another valuable characteristic of novel biomarkers. To assess this issue, in a second validation step, we studied the expression of our panel of miRNAs in a cohort of patients which underwent cardiac surgery with CPB. This experimental design offers a context where the moment and the severity of kidney insult is known and patient outcome can be closely followed. Several biomarkers have been tested in this clinical context to predict AKI development immediately or few hours after intervention. They have demonstrated very high predictive values in pediatric populations but more variable results in adults. NGAL presented AUC values ranging from 0.54 to 0.87, IL-18 from 0.55 to 0.87, Cystatin-C from 0.73 to 0.76 and from 0.68 to 0.78 in the case of KIM-1 (For review Mariscalco G. et al., 2011; Siew ED. et al., 2011).

Our results demonstrated that basal levels of miR-26b, miR-27a, miR-93* and miR-127-3p, measured before surgery, can robustly predict AKI development after CPB, independently of the AKI criteria used for diagnosis. They show a very high predictive potential with AUC values comprised between 0.7 and 0.9. This evidence demonstrates that several miRNAs included in our panel are robust markers of AKI predisposition. Only KIM-1 has demonstrated this ability by predicting stage 3 AKI after CPB with an AUC value of 0.8 (Koyner JL. et al., 2010). However, miRNAs can predict AKI before surgery independently of its severity. This worthy information could have a great impact in clinical practice by allowing the detection of patients with risk of AKI development after surgery and the application of prevention strategies.

However, these high predictive values are not observed in serum miRNAs determination immediately after surgery, when AUC values obtained vary from 0.6 to 0.7. This drastic change in discriminative ability is probably due to the dilution factor produced by the intense hydration

treatment to which operated patients are submitted during surgery, probably requiring a correction factor, such as normalization by hematocrit.

In addition, our correlation studies demonstrate that miR-26b, miR-146a, miR-93* and miR-127-3p progressively decrease their expression levels in serum during the days prior to AKI diagnosis by serum creatinine. These data demonstrate that serum miRNAs can detect AKI development several days before serum creatinine. It is important to notice that miR-146a, which demonstrated a very high diagnostic potential in ICU patients, presents here a strong and significant downregulation during previous days to AKI. These data confirm that this miRNA could be considered as a robust and early AKI diagnostic tool in several clinical contexts.

As indicated in previous sections, AKI is a dynamic syndrome which includes progressive structural and functional damage and whose appropriate treatment requires a complete information collection from early injury to long-term outcomes.

Based on this dynamic view of AKI, Pickering and Endre developed the concept of Phase-Specific AKI Biomarkers. Phase-1 Biomarkers identify risk of AKI allowing the application of preventive strategies. In this regard, our panel of miRNAs has demonstrated the ability to identify risk patients before surgery. On the other hand, Phase 2 Biomarkers are important for injury identification, which is essential to precipitate early therapeutic interventions. Our data demonstrate that miRNAs progressively decrease their expression levels in serum days before AKI diagnosis by creatinine. Phase 3 Specific Biomarkers are able to monitor injury and repair. In this regard, our results indicate that miRNAs expression levels correlate with severity of renal injury and they also indicate different recovery degrees. Finally, Phase 4 Specific Biomarkers permit the monitoring of AKI outcomes (Pickering JW and Endre ZH, 2009b). Our data suggest that serum miRNAs could indicate a possible long-term evolution to other renal pathologies, particularly CKD. Other outcomes such as mortality or RRT requirement need to be further investigated in following studies.

Based on the above discussed features, miRNAs have been unveiled as complete and dynamic biomarkers able to detect almost all the structural and functional features included in the complex pathophysiology of AKI, from risk identification to long-term evolution or outcome.

Despite all these promising results, the present work presents several limitations that should be solved in further studies. First, this is a pilot study with a limited number of patients. Further studies should be done increasing the number of patients included in each context and in collaboration with other hospitals to confirm these data and to give an increased statistical

significance to our results. Moreover, prospective studies need to be performed to explore the association of miRNA expression with worse prognosis and outcomes such as RRT requirement or mortality. Prognostic value such as acute-on-chronic kidney disease prediction, kidney function after renal transplant or monitoring responses to therapies needs to be also investigated.

On the other hand, as in the majority of AKI biomarker studies, serum creatinine has been used as the gold standard for AKI diagnosis in this work. Despite the widely known limitations of this marker and the multifaceted nature of AKI, changes in serum creatinine levels remains the main criteria for phenotyping this disease. Therefore, in the absence of other potentially more informative standard such as tissue examination, the use of creatinine as a comparator is not unreasonable, as we have done in this work.

It is important to notice that comparison with creatinine information is not criteria enough to accept or reject a new biomarker, since new biomarkers are indeed providing new information that can be initially difficult to understand and interpret. Indeed, recent works are pointing out the existence of subclinical AKI episodes in which kidney suffers structural but not functional damage (Murugan R and Kellum JA, 2011). These cases are currently undetected by serum creatinine and could be determinant for future more severe AKI episodes or chronic diseases. New biomarkers could contribute to improve subclinical AKI diagnosis indicating renal damage without increase in creatinine levels.

On the other hand, current biomarker studies rely on ROC statistical analysis to assess discriminative ability and diagnostic potential. However, several works have reported that AUC-ROC values are a relatively insensitive metric since extremely robust associations between the outcome and minimal overlapping are required to significantly improve AUC values. Based on this feature, increasing evidence suggest that novel statistical prediction models to ascertain clinical value could be needed for these studies (Pencina MJ. et al., 2008; Pickering JW and Endre ZH, 2012).

Although miRNAs present a very high diagnostic value by themselves, several studies have pointed out that combination of several biomarkers significantly improves diagnostic precision. For instance, combination of NGAL, NAG and KIM-1 in a logistic regression model increases predictive ability from individual AUC values around 0.6 to 0.78 in the combined model (Han WK et al., 2009). In this regard, biomarkers which are generated in a similar biological pathway can present a very high probability of colinearity (Siew ED, et al., 2011). Due to this, a combination of biomarkers, including miRNAs, with different biological properties and origin and matching different pathophysiological pathways could avoid information overlapping offering integrated diagnostic information of the pathology.

Despite the advantages of all the biomarkers currently in development, none of them have demonstrated yet a clear and unmistakable clinical advantage compared to serum creatinine. Thus, novel biomarkers could be considered as a tool for complementing serum creatinine rather than to replace it. Novel future AKI classifications have been proposed in which two domains can be found, one for function measuring, based on serum creatinine, and the other for damage estimation based on changes of a panel of biomarkers. In this theoretical classification, 'risk' stage is determined by small changes in one marker while 'failure' might require more than one changes in several markers or possibly large changes in a single marker (Murugan R and Kellum JA, 2011).

In summary, in this work we identified for the first time a novel role of miR-127 as a critical regulator of cell-cell and cell-matrix adhesion in proximal tubule cells response to I/R. Additionally we unveiled a new regulation of this miR-127 through HIF-1α. Moreover, a novel target gene for this miRNA was also elicited: KIF3B, with important implications in cell endocytosis. As cell adhesion and cell trafficking are essential for proximal tubule epithelial structure and function, miR-127 and KIF3B could be considered as key molecules for renal ischemic damage management. In addition, a panel of serum microRNAs, including miR-127, has been unveiled as novel biomarkers of AKI. Serum miRNAs present a high diagnostic potential in ICU patients and their expression levels correlate with AKI severity. Serum miRNAs could be also potential biomarkers of different recovery degrees and differentiate between AKI etiologies and pre-renal or intrinsic origin. Indeed, serum miRNAs associated to ischemic AKI can determine AKI predisposition in a cohort of patients submitted to CPB. Serum levels of these miRNAs progressively decrease days before AKI diagnosis, indicating their potential as early injury biomarkers.

This work constitutes an example of translational research from bench to the bedside in which molecular mechanism of AKI studied in experimental *in vitro* and *in vivo* models, can be useful in clinical context. Thus, miR-127 has been identified in *vitro* and *in vivo* as a key mediator of renal responses to I/R injury and is also a biomarker of ischemic AKI showing a high early diagnostic and predisposition identification value. It is important to notice that, through intellectual property application for the miRNA combination identified and validated here, we are trying to generate an AKI diagnostic kit for clinical use.

CONCLUSIONS

1. rno-miR-127 and its human homologous hsa-miR-127-3p are induced in an *in vitro* model of Hypoxia/Reoxygenation (H/R) in proximal tubule cells and in an *in vivo* model of renal ischemia/Reperfusion (I/R) in rat. This induction is regulated by HIF-1α, among other mechanisms.

2. rno-miR-127 induction during H/R prevents actin depolimerization and cell adhesion alterations by reducing Focal Adhesion Complexes disassembly and Tight Junction disorganization.

3. KIF3B is a real target of rno-miR-127 in proximal tubule cells during H/R, with important implications in proximal tubule cell trafficking.

4. Serum miRNAs miR-127-3p, miR-101-1, miR-210, miR-126, miR-26b, miR-29a, miR-146a, miR-27a, miR-93* and miR-10a are diagnostic biomarkers of AKI in ICU patients compared to healthy controls.

5. miR-210, miR-126, miR-29a and miR-146a expression levels correlate with AKI severity and can discriminate between pre-renal and intrinsic AKI.

6. miR-26b, miR-146a, miR-93* and miR-127-3p indicate AKI development before serum creatinine increases, becoming as early biomarkers for AKI diagnosis in a cohort of patients submitted to cardiac surgery with cardiopulmonary bypass.

7. miR-26b, miR-27a, miR-93* and miR-127-3p, estimated in serum samples obtained before cardiac surgery, are biomarkers of AKI predisposition in this clinical situation.

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PUBLICATIONS, PATENTS AND MEETINGS

ARTICLES

The data described in this work have generated the following articles:

AUTHORS: **Elia Aguado-Fraile**, Edurne Ramos, David Sáenz-Morales, Elisa Conde, Ignacio Blanco-Sánchez, Konstantinos Stamatakis, Luis del Peso, Edwin Cuppen, Bernhard Brüne and María Laura García Bermejo.
TITLE: miR-127 protects proximal tubule cells against ischemia/reperfusion: identification of Kinesin Family member 3B as miR-127 target
JOURNAL: **PLoS One.** 2012;7(9):e44305
Impact factor: **4.35**.

AUTHORS: **Elia Aguado-Fraile**, Edurne Ramos, Elisa Conde, Macarena Rodríguez, Aurora Lietor, Teresa Tenorio, Angel Candela, Fernando Liaño and M Laura García-Bermejo.
TITLE: Serum microRNAs are accurate biomarkers of Acute Kidney Injury in ICU patients.
(In preparation).

AUTHORS: **Elia Aguado-Fraile**, Edurne Ramos, Elisa Conde, Macarena Rodríguez, Fernando Liaño, Elena Elías Martín, Ángel Candela, M Laura García-Bermejo.
TITLE: Serum microRNAs: biomarkers of AKI predisposition and diagnosis after cardiac surgery.
(In preparation).

Moreover, the author has contributed to the following publications:

AUTHORS: Leticia Muñoz, María José Borrero, María Ubeda, Margaret Lario, David Díaz, Rubén Francés, Jorge Monserrat, Óscar Pastor, José Such, **Elia Aguado**, Melchor Álvarez-Mon, Agustín Albillos.
TITLE: Interaction Between Intestinal Dendritic Cells and Bacteria Translocated from the Gut in Rats with Cirrhosis
JOURNAL: **Hepatology**. 2012 May 21. doi: 10.1002/hep.25854.
Impact factor: **10,885**.

AUTHORS: Elisa Conde, Laura Alegre, Ignacio Blanco-Sánchez, David Sáenz-Morales, Ana Sáiz, Belén Ponte, **Elia Aguado**, Edurne Ramos, Carlos Jiménez, Ángel Ordoñez, Manuel Ortiz de Landazuri, Manuel López-Cabrera, Luis del Peso, Fernando Liaño, Rafael Selgas, Jose Antonio Sanchez-Tomero and Maria Laura García-Bermejo.
TITLE: "Hypoxia Inducible Factor 1-alpha (HIF-1 alpha) is induced during reperfusion after renal ischemia and is critical for proximal tubule cell survival."
JOURNAL: **PLoS One**. 2012;7(3):e33258.
Impact factor: **4,35.**

AUTHORS: Ana Belén Fernández-Martinez, María Isabel Arenas-Jiménez, Irene Sánchez- Hernández, María Laura García-Bermejo, Victoria Moreno- Manzano, **Elia Aguado**, Francisco Javier de Lucio-Cazaña
TITLE: "Hypoxia-inducible factor-1α-dependent regulation of retinoic acid receptor-beta in human proximal tubular cells HK-2"
JOURNAL: **Int J Biochem Cell Biol.** 2011 Aug;43(8):1198-207.
Impact factor: **4,8**

AUTHORS: Sáenz-Morales D, Conde E, Blanco-Sánchez I, Ponte B, **Aguado-Fraile E**, de Las Casas G, García-Martos M, Alegre L, Escribese MM, Molina A, Santiuste C, Liaño F, García-Bermejo ML.
TITLE: Differential resolution of inflammation and recovery after renal ischemia–reperfusion injury in Brown Norway compared with Sprague Dawley rats
JOURNAL: **Kidney Int.** 2010 May;77(9):781-93. Epub 2010 Feb 17.
Impact factor: **6.418**.

PATENTS

The data described in this work have generated the following Patent Applications:

Inventors: **Aguado Fraile Elia**, Liaño García Fernando, Sáenz Morales David y Garcia-Bermejo, M. L
Title: Método de diagnóstico y/o pronóstico del daño renal agudo.
Application number: **200901825 / PCT ES10070579**
Priority countries: **España, extensión PCT Entrada en fases nacionales en Brasil, Canadá, Europa, India, Australia, China, Rusia, Japón, Mexico y USA.**
Application date: 04/09/2009.
Date of approval in Spain: 18/05/2012
Entity holding: Fundación para la Investigación Biomédica del Hospital Ramón y Cajal.

Inventors (p.o. de firma): **Aguado Fraile Elia**, Liaño García Fernando, Sáenz Morales David y Garcia Bermejo, M. L
Title: Método de diagnóstico y/o pronóstico de daño renal agudo.
Application number: **P201130546 (divisional de la solicitud prioritaria P200901825) / PCT ES10070579**
Priority countries: **España, extensión PCT Entrada en fases nacionales en Brasil, Canadá, Europa, India, Australia, China, Rusia, Japón, Mexico y USA.**
Divisional application date: 06/04/2011
Entity holding: Fundación para la Investigación Biomédica del Hospital Ramón y Cajal.

Inventors (p.o. de firma): **Aguado Fraile Elia**, Liaño García Fernando, Sáenz Morales David y Garcia Bermejo, M. L
Title: Método de diagnóstico y/o pronóstico de daño renal agudo.
Application number: **P201130545 (divisional de la solicitud prioritaria P200901825) / PCT ES10070579**
Priority countries: **España, extensión PCT Entrada en fases nacionales en Brasil, Canadá, Europa, India, Australia, China, Rusia, Japón, Mexico y USA.**
Divisional application date: 06/04/2011
Entity holding: Fundación para la Investigación Biomédica del Hospital Ramón y Cajal.

Inventors (p.o. de firma): **Aguado Fraile Elia**, Ramos Muñoz Miren Edurne, Candela Toha Angel Manuel, Liaño García Fernando y Garcia Bermejo, M. L
Title: Método para el diagnóstico y/o pronóstico del daño renal agudo
Application number: **P201132023**
Priority countries: **España**
Application date: 15/12/2011
Entity holding: Fundación para la Investigación Biomédica del Hospital Ramón y Cajal.

COMMUNICATIONS TO SCIENTIFIC MEETINGS

The data described in this work and collaborations have generated the following congress communications:

INTERNATIONAL MEETINGS

Authors: **Elia Aguado-Fraile,** Edurne Ramos, Elisa Conde, Macarena Rodríguez, Aurora Lietor, Teresa Tenorio, Angel Candela, Fernando Liaño and M Laura García-Bermejo.
Title: **"Serum microRNAs are accurate biomarkers of Acute Kidney Injury in ICU patients"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 45th Annual Meeting
Venue: San Diego, CA, USA
Date: October 30th-Noverber 4th, 2012

Authors: **Elia Aguado-Fraile**, Edurne Ramos, Elisa Conde, Macarena Rodríguez, Fernando Liaño, Elena Elías Martín, Ángel Candela, M Laura García-Bermejo.
Title: **"Serum microRNAs are predictive biomarkers of Acute Kidney Injury after Cardiac surgery"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 45th Annual Meeting
Venue: San Diego, CA, USA
Date: October 30th-Noverber 4th, 2012

Authors: Elisa Conde, Edurne Ramos, Ignacio Blanco-Sánchez, **Elia Aguado-Fraile**, Macarena Rodríguez, María Laura García Bermejo
Title: **"HIF-1α promotes renal tissue repair after ischemic damage controlling proximal tubule cell proliferation, cell death and inflammatory response"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 45th Annual Meeting
Venue: San Diego, CA, USA
Date: October 30th-Noverber 4th, 2012

Authors: Patricia Corrales-Cordón, Hector Pian*, **Elia Aguado-Fraile*,** Elisa Conde, Edurne Ramos, Macarena Rodriguez, Concepción Sánchez, Eva María Guerra, Augusto García, J Fernando González Palacios, Alejandro Pascual and M Laura García-Bermejo.
* Equal contribution
Title: **"miRNAs as novel molecular markers for breast cancer diagnosis"**
Type of presentation: Poster
Congress: 22nd IUBMB & 37th FEBS Congress
Venue: Sevilla, Spain
Date: September 4-9th, 2012

Authors: **Elia Aguado-Fraile**, Edurne Ramos, David Sáenz-Morales, Elisa Conde, Ignacio Blanco-Sánchez, Konstantinos Stamatakis, Bernhard Brüne and María Laura García Bermejo.
Title: **miR-127 induced during renal ischemia/reperfusion via HIF-1α protects proximal tubule cells against ischemic injury: KI3FB as miR-127 target**
Type of presentation: Oral communication
Congress: HypoxiaNet: Learning for Hypoxia Signalling
Venue: Bilbao, Spain
Date: April 18-20th, 2012

Authors: Elisa Conde, Laura Alegre, Ignacio Blanco-Sánchez, David Sáenz-Morales, Ana Sáiz, **Elia Aguado**, Edurne Ramos, Fernando Liaño, and María Laura García Bermejo
Title: **"Hypoxia Inducible Factor 1-alpha (HIF-1 alpha) is induced during reperfusion after renal ischemia and is critical for proximal tubule cell survival."**
Type of presentation: Poster
Congress:HypoxiaNet: Learning for Hypoxia Signalling
Venue: Bilbao, Spain
Date: April 18-20th, 2012

Authors: C Guillén-Ponce, E. Conde, **E. Aguado-Fraile**, E. Ramos, A. Carrato and ML. García-Bermejo.
Title: **Genetic differences based on miRNAs of colorectal adenocarcinoma according to age and the presence or absence of MMR defect.**
Type of presentation: Poster
Congress: 2012 Gastrointestinal Cancers Symposium
Venue: San Francisco, USA
Date: January 19-21th, 2012

Authors: **E. Aguado-Fraile**, E. Ramos, N. Villegas, E. Conde, I. Blanco-Sánchez, A Candela, F. Liaño and ML. García-Bermejo.
Title: **"miR−127 Regulates Trafficking in Proximal Tubule Cells Trough Its Target KIF3B in Response to Ischemia/Reperfusion"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 44th Annual Meeting
Publication:
Venue: Philadelphia, Pennsylvania, USA
Date: November 18-23th, 2011

Authors: **E. Aguado-Fraile**, E. Ramos, N. Villegas, F. Diaz, E. Conde, I. Blanco-Sánchez, A Candela, F. Liaño and ML. García-Bermejo.
Title: **"Identification and Validation of microRNAs as New Biomarkers of Acute Renal Failure"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 44th Annual Meeting
Publication:
Lugar celebración: Philadelphia, Pennsylvania, USA
Date: November 18-23th, 2011

Authors: Ignacio Blanco-Sánchez, Marina Arranz, Elisa Conde, **Elia Aguado**, Edurne Ramos, Rafael Selgas, J. Antonio Sánchez-Tomero y M Laura García-Bermejo.
Title: **"Signalling Pathways Responsible for Proximal Tubule Cell Adhesion Promotion during I/R by Darbepoetin a: New Therapeutic Targets for a New Use of Darbepoetin α"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 44th Annual Meeting
Venue: Philadelphia, Pennsylvania, USA
Date: November 18-23th, 2011

Authors: **E. Aguado-Fraile**, E. Ramos, D. Sáenz-Morales, E. Conde, I. Blanco-Sánchez, M. Martínez-Colmenar, F. Liaño and ML. García-Bermejo.
Title: **"miR-127-3p induced by HIF-1a is a cytoskeleton protector mechanism in the proximal tubule response to ischemia/reperfusion"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 43rd Annual Meeting
Venue: Denver, Colorado, USA
Date: November 16-21th, 2010

Authors: Elisa Conde, Ignacio Blanco-Sánchez, **Elia Aguado**, Marta Martínez, Edurne Ramos, J Antonio Sánchez-Tomero, Rafael Selgas, M Laura García-Bermejo.
Title: **"HIF induction during reperfusion is critical for kidney regeneration after renal ischemia"**
Type of presentation: **Oral communication**
Congress: American Society of Nephrology, ASN 43rd Annual Meeting
Lugar celebración: Denver, Colorado, USA
Date: November 16-21th, 2010

Authors: Ignacio Blanco-Sánchez, Elisa Conde, **Elia Aguado**, Marta Martínez, Edurne Ramos, Rafael Selgas, J. Antonio Sánchez-Tomero y M Laura García-Bermejo.
Title: **" Benefitial effects of Darbepoietin-alpha administration in renal ischemic injury: promotion of tubular adhesion"**
Type of presentation: Poster
Congress: American Society of Nephrology, ASN 43rd Annual Meeting
Venue: Denver, Colorado, USA
Date: November 16-21th, 2010

Authors: I. Blanco-Sánchez, E. Conde, D. Sáenz-Morales, **E. Aguado**, Y. Rodríguez, R. Selgas, J.A. Sánchez-Tomero and ML. García-Bermejo.
Title: **Dual effect of DarbepoietinTM administration dose dependent during renal ischemia: protection or regeneration.**
Type of presentation: Póster
Congress: American Society of Nephrology, ASN 42Th Annual Meeting
Venue: San Diego, USA
Date: October 29th-November 1st, 2009

NATIONAL MEETINGS

Authors: E. Conde Moreno, **E. Aguado**, J. Soto, C. Alenda, A. Castillejo, V. M. Barbera, D. Salas, A. Carrato, M. L. Garcia Bermejo, Carmen Guillén Ponce
Title: **Expresión diferencial de miRNAs en adenocarcinoma colorrectal según la edad y la presencia o ausencia de defecto MMR: nuevos marcadores diagnósticos y dianas de actuación terapéutica**
Type of presentation: **Oral communication**.
Congress: 2º Simposio Nacional Sociedad Española de Oncología Médica (SEOM)
Venue: Madrid, España
Date: October 24-26th, 2012

Authors: L. Muñoz, M.J. Borrero, M. Ubeda, M. Lario, D. Díaz, **E. Aguado**, L. García Bermejo, L. Lledó, M. Álvarez-Mon, A. Albillos.
Title: **Contribución de la IL15 al establecimiento de la inflamación intestinal en ratas con cirrosis**
Type of presentation: Póster.
Congress: V Jornadas científicas de CIBERehd, 2011
Venue: Barcelona, España
Date: November 22-23th , 2011

Authors: **Elia Aguado-Fraile**, Francisco Díaz-Crespo, Edurne Ramos, Nuria Villegas, Elisa Conde, Ignacio Blanco-Sánchez, Marina Arranz, Ángel Candela, Teresa Tenorio, Fernando Liaño, M Laura García-Bermejo
Title: **Identificación y validación de micrornas como nuevos biomarcadores de daño renal agudo.**
Type of presentation: **ORAL COMMUNICATION**
Congress: XLI Congress Nacional de la Sociedad Española de Nefrología
Venue: Sevilla, España
Date: October 15-18th, 2011

Authors: **Elia Aguado***, Ana Saiz*, David Sáez Morales, Elisa Conde, Ignacio Blanco-Sánchez, Yaiza Rodríguez, Eva Cristóbal, Adrián Cuevas, Ricardo García González, Fernando González Palacios y María Laura García Bermejo
Title: **Determinación de la expresión de miRNAs y sus dianas como nuevos marcadores pronósticos de progresión en el carcinoma renal.**
Type of presentation: Poster
Congress: Reunión Anual de la Sociedad Española de Anatomía Patológica
Venue: Sevilla, España
Date: May 18th, 2009

Authors: Elisa Conde*, Ana Saiz*, Laura Alegre, Ignacio Blanco-Sánchez, David Sáez Morales, Carlos Jiménez, **Elia Aguado**, Yaiza Rodríguez, Fernando González Palacios, J Antonio Sánchez- Tomero, Rafael Selgas y María Laura García Bermejo.
Title: **Detección del factor inducible por hipoxia (HIF-1a) y sus genes diana en biopsias de trasplante renal como indicadores de reparación de NTA.**
Type of presentation: Poster
Congress: Reunión Anual de la Sociedad Española de Anatomía Patológica
Venue: Sevilla, España
Date: May 18th, 2009

Authors: Ignacio Blanco-Sánchez*, Ana Saiz*, Elisa Conde, David Sáez Morales, **Elia Aguado** Yaiza Rodríguez, Fernando González Palacios, Rafael Selgas, José Antonio Sánchez Tomero y María Laura García Bermejo
Title: **Valoración de la progresión del daño isquémico renal en un modelo experimental de I/R en ratas tratadas con Darbepoietina.**
Type of presentation: Póster
Congress: Reunión Anual de la Sociedad Española de Anatomía Patológica
Venue: Sevilla, España
Date: May 18th, 2009

ANNEXES

ANNEX 1: Informed consent document

HOJA DE INFORMACIÓN AL PACIENTE Y CONSENTIMIENTO INFORMADO PARA MUESTRAS DE SANGRE Y ORINA

Título del estudio: Identificación y validación de microRNAs como nuevos biomarcadores de evolución del daño renal isquémico tras cirugía cardiaca y en el trasplante renal. Estudio de su potencial terapéutico.

Investigador principal
Dra. Maria Laura García Bermejo
Servicio de Anatomia Patológica, Laboratório Respuesta Celular a la Isquemia,
Planta -3 izda
Hospital Ramón y Cajal
Tfno: 91 336 80 75 / 699 134 789
Email: mgarciab.hrc@salud.madrid.org

INTRODUCCIÓN

Nos dirigimos a usted para informarle sobre el desarrollo del estudio en el que se le propone participar Nuestra intención es tan solo que usted reciba la información correcta y suficiente para que pueda evaluar y juzgar si quiere o no participar en este estudio. Para ello lea esta hoja informativa con atención y nosotros le aclararemos las dudas que le puedan surgir después de la explicación.
Su participación es voluntaria y puede revocar su decisión y retirar el consentimiento en cualquier momento sin que por ello se altere la relación con su médico ni se produzca perjuicio en sus cuidados médicos. En caso de retirar el consentimiento para participar en el estudio, ningún dato nuevo será añadido a la base de datos y puede exigir la destrucción de todas las muestras identificables previamente retenidas para evitar la realización de un nuevo análisis.

FUNDAMENTO

El estudio que se va a llevar a cabo es un estudio promovido por la **Dra. García Bermejo,** con el fin de **identificar y validar microRNAs como nuevos biomarcadores de evolución del daño renal isquémico en casos de cirugía cardiaca y de trasplante renal , así como explorar su potencial terapéutico.**

El objetivo final del estudio es generar nuevos y más precisos y precoces biomarcadores del daño renal y para ello se va a realizar, una extracción de RNA de parte de las muestras de sangre y orina que se le requerirán con fines diagnósticos y clínicos. Ese RNA será amplificado por técnicas de RT-PCR para detectar la presencia de los mRNAs: mir-127, miR-101, miR-126 y miR-210. Esto **NO SUPONE** realizarle nuevos pinchazos aparte de los que precisa para su cuidado y que en cualquier caso le indicará su médico. La evolución en los niveles de estos miRNAs en sus muestras de sangre y orina será contrastada con otros parámetros de uso clínico habitual.

Cualquier nueva información referente a las técnicas utilizadas en el estudio, que se descubra durante su participación, le será comunicada.

BENEFICIOS ESPERADOS E INCONVENIENTES

El beneficio esperado con este estudio es validar el uso clínico de unos nuevos marcadores de daño renal por isquemia, como el que sucede tras cirugía cardiaca o en el trasplante renal, que definan de forma más precoz y precisa que compartimento celular del riñón se está dañando. Al marcar el daño más tempranamente y además definir qué parte está dañada, se podría aplicar terapéutica eficaz mucho más tempranamente y resolver la disfunción renal antes, con todo los beneficios que ello conlleva como evitar la diálisis en algunos casos. Por otro lado este estudio quiere explorar la posibilidad de que estos miRNAs puedan ser de utilidad terapéutica.

CONFIDENCIALIDAD

Este estudio **NO REQUIERE** recoger más datos que los habituales.

En cualquier caso, todos los datos recogidos para el estudio, procedentes de su Historia Clínica o facilitados por usted mismo, serán tratados con las medidas de seguridad establecidas en cumplimiento de la Ley Orgánica 15/1999 de Protección de Datos de carácter personal. Debe saber que tiene derecho de acceso rectificación y cancelación de los mismos en cualquier momento. Sólo aquellos datos de la historia clínica que estén relacionados con el estudio serán objeto de comprobación. Esta comprobación la realizará e

Investigador Principal/Investigadores Colaboradores, responsables de garantizar la confidencialidad de todos los datos de las historias clínicas pertenecientes a los sujetos participantes en el estudio. Los datos recogidos para el estudio estarán identificados mediante un código y sólo el investigador principal/colaboradores podrán relacionar dichos datos con usted y con su historia clínica.

Las muestras, al ser depositadas en el Biobanco-FRA del Hospital Universitario Ramón y Cajal van a ser **ANONIMIZADAS** Todas las muestras que se obtengan para el estudio (sangre y orina) serán codificadas de forma que no sea posible su identificación y no será posible establecer de nuevo el nexo con sus datos personales, siguiendo los procedimientos habituales de almacenamiento y registro de dicho Biobanco.

Nuevos análisis: El derecho del sujeto de estar informado de cualquier proyecto de nuevos análisis de material identificable retenido que no fue previsto cuando el sujeto dio su consentimiento para participar en el estudio. El investigador debe pedir un nuevo consentimiento y el sujeto tiene el derecho de rehusar más análisis, conforme a la legislación nacional

:

OTROS ASPECTOS DE INTERÉS

Algunos de los datos obtenidos con el uso de las muestras recogidas podrían formar parte de una Patente de USO de miRNAs como marcadores de daño isquémico renal que se está tramitando

En caso de necesitar más información sobre el estudio o cualquier otra duda que pueda tener, no dude en contactar con cualquiera de los investigadores del estudio o con el Biobanco del Hospital Universitario Ramón y Cajal en el teléfono: 91 336 79 55 o el e-mail: biobanco@salud.madrid.org. Así mismo, en caso de dudas respecto a sus derechos puede dirigirse al Servicio de Atención al Paciente del hospital.

Firma del paciente:	**Firma del investigador que le facilita la información**:
Nombre: **Fecha**:	**Nombre**: **Fecha**:

Este documento se firmará por duplicado quedándose una copia el investigador y otra el paciente

MODELO DE CONSENTIMIENTO INFORMADO POR ESCRITO

Título del Estudio: Identificación y validación de microRNAs como nuevos biomarcadores de evolución de daño renal isquémico tras cirugía cardiaca y en el trasplante renal. Estudio de su potencial terapéutico.

Código de protocolo:

Promotor: Dra. María Laura García Bermejo

Yo (nombre y apellidos)

.....

He leído la hoja de información que se me ha entregado.

He podido hacer preguntas sobre el estudio.

He recibido suficiente información sobre el estudio.

He hablado con:

Dr/Dra Investigador colaborador del estudio

Comprendo que mi participación es voluntaria.
Comprendo que puedo retirarme del estudio:
1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en mis cuidados médicos.
Presto libremente mi conformidad para participar en el estudio.

FECHA : FIRMA DEL PARTICIPANTE

FECHA : FIRMA DEL INVESTIGADOR

MODELO DE CONSENTIMIENTO INFORMADO POR ESCRITO

Título del Estudio: Identificación y validación de microRNAs como nuevos biomarcadores de evolución del daño renal isquémico tras cirugía cardiaca y en el trasplante renal. Estudio de su potencial terapéutico.

Código de protocolo:

Promotor: Dra. María Laura García Bermejo

Yo (nombre y apellidos)

.....

He leído la hoja de información que se me ha entregado.
He podido hacer preguntas sobre el estudio.
He recibido suficiente información sobre el estudio.
He hablado con:
Dr/Dra Investigador colaborador del estudio

Comprendo que la participación del paciente es voluntaria.
Comprendo que puede retirarse del estudio:
1º Cuando quiera
2º Sin tener que dar explicaciones.
3º Sin que esto repercuta en sus cuidados médicos.
Presto libremente mi conformidad para participar en el estudio.

FECHA: FIRMA DEL REPRESENTANTE LEGAL/TESTIGO

FECHA: FIRMA DEL INVESTIGADOR

ANNEX 2: Plos One Publication

OPEN ACCESS Freely available online

PLOS ONE

miR-127 Protects Proximal Tubule Cells against Ischemia/Reperfusion: Identification of Kinesin Family Member 3B as miR-127 Target

Elia Aguado-Fraile[1], Edurne Ramos[1], David Sáenz-Morales[1], Elisa Conde[1], Ignacio Blanco-Sánchez[1], Konstantinos Stamatakis[2], Luis del Peso[3,4], Edwin Cuppen[5], Bernhard Brüne[6], María Laura García Bermejo[1,7]*

1 Department of Pathology, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain, **2** Department of Cell Biology and Immunology, Centro de Biología Molecular Severo Ochoa (CBM-SO) (CSIC-UAM), Madrid, Spain, **3** Department of Biochemistry/HIV Unit, Hospital La Paz (IdiPAZ), Madrid, Spain, **4** Institute of Biomedical Research Alberto Sols, CSIC-UAM, Madrid, Spain, **5** Genome Biology Group, Hubrecht Institute, Utrecht, The Netherlands, **6** Pathobiochemistry, Faculty of Medicine, Goethe-University Frankfurt, Frankfurt, Germany, **7** Physiology Department, Alcalá University, Madrid, Spain

Abstract

Ischemia/reperfusion (I/R) is at the basis of renal transplantation and acute kidney injury. Molecular mechanisms underlying proximal tubule response to I/R will allow the identification of new therapeutic targets for both clinical settings. microRNAs have emerged as crucial and tight regulators of the cellular response to insults including hypoxia. Here, we have identified several miRNAs involved in the response of the proximal tubule cell to I/R. Microarrays and RT-PCR analysis of proximal tubule cells submitted to I/R mimicking conditions *in vitro* demonstrated that miR-127 is induced during ischemia and also during reperfusion. miR-127 is also modulated in a rat model of renal I/R. Interference approaches demonstrated that ischemic induction of miR-127 is mediated by Hypoxia Inducible Factor-1alpha (HIF-1α) stabilization. Moreover, miR-127 is involved in cell-matrix and cell-cell adhesion maintenance, since overexpression of miR-127 maintains focal adhesion complex assembly and the integrity of tight junctions. miR-127 also regulates intracellular trafficking since miR-127 interference promotes dextran-FITC uptake. In fact, we have identified the Kinesin Family Member 3B (KIF3B), involved in cell trafficking, as a target of miR-127 in rat proximal tubule cells. In summary, we have described a novel role of miR-127 in cell adhesion and its regulation by HIF-1α. We also identified for the first time KIF3B as a miR-127 target. Both, miR-127 and KIF3B appear as key mediators of proximal epithelial tubule cell response to I/R with potential al application in renal ischemic damage management.

Citation: Aguado-Fraile E, Ramos E, Sáenz-Morales D, Conde E, Blanco-Sánchez I, et al. (2012) miR-127 Protects Proximal Tubule Cells against Ischemia/Reperfusion: Identification of Kinesin Family Member 3B as miR-127 Target. PLoS ONE 7(9): e44305. doi:10.1371/journal.pone.0044305

Editor: Wing-ho Yung, The Chinese University of Hong Kong, Hong Kong

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Competing Interests: The authors have declared that no competing interests exist.

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Introduction

microRNAs (miRNAs) are small (~21 nucleotide-long), endogenous RNA molecules that have emerged as key post-transcriptional regulators of gene expression [1]. They are involved in a wide range of biological processes, including development, cell proliferation and differentiation, apoptosis and metabolism [1–3]. Bioinformatics approaches have described that, in mammals, they could regulate almost ~50% of the protein-coding genes [1] and changes in their expression have been related to the pathogenesis of several human diseases [3].

In animals, most miRNAs are processed from longer hairpin transcripts by the action of two members of the RNAse III family of enzymes called Drosha and Dicer. This cleavage generates a ~20 nucleotide miRNA/miRNA* duplex. One strand of the hairpin duplex is loaded into an Argonaute Family Protein (AGO) to form the miRNA-Induced silencing complexes (miRISCs) [1,3]. As a part of these complexes, miRNAs silence the expression of target genes by translational repression or mRNA deadenylation and degradation [1]. Due to their ability to recognize hundreds of target mRNA and their reversible regulation, miRNAs have emerged as key controllers of rapid cell responses to environmental changes and stress [1,4].

Ischemia/Reperfusion is one of the principal causes of Acute Tubular Necrosis, which underlies most of the cases of Acute Renal Failure. Sublethal ischemic injury is characterized by a rapid loss of proximal tubule cell polarity and cytoskeleton integrity. After ischemia, apical actin cytoskeleton is rapidly reorganized and adhesion molecules change their localization. These features lead to impairment of cell-cell and cell-matrix adhesion structures and cell detachment and consequently kidney dysfunction [5–8].

HIF-1α is a key modulator of cellular transcriptional response to low oxygen conditions and it activates a great number of metabolic and bioenergetic adaptative responses to hypoxic conditions [9].